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BIOLOGICALLY ACTIVE PEPTIDE CONJUGATES

FIELD OF INVENTION

The present invention is related to the field of immunology. In particular, the present invention is directed to peptides and their pharmaceutical compositions which are capable of modulating immune responses.

BACKGROUND OF INVENTION

Spleen extracts from mammals have been used successfully for years as medical immune modulators. However, the molecular composition of such extracts is unknown and therefore the exact pharmacological actions of these extracts cannot be identified. The dosage response function also cannot be accurately titrated because of the intrinsic heterogeneous nature of these extracts. Because of such heterogeneity, active ingredients in spleen extracts cannot be singled out and their pharmacological actions cannot be fully utilized. And, no matter how careful the extraction process can be, there is always the possibility of transmission of diseases of animal origin to human if animal tissue is used as the raw material for extraction.

US Patent No. 3,992,364 to Kuhlmey describes a physiologically active polypeptide produced from an animal spleen extract. The exact sequence of the peptide was not disclosed and the preparation was described as being useful for decreasing cholesterol levels and increasing the 17 keto-steroid elimination of human patients.

SUMMARY OF INVENTION

It is an object of the present invention to identify biologically active polypeptides.

A study was conducted in which a number of peptides have been identified from a porcine spleen extract according to the method described in US 3,992,364. . The peptides in the extract were separated by preparative HPLC followed by analysis using electrospray ionisation mass spectroscopy (ESMS) and matrix assisted laser desorption ionisation mass spectroscopy (MALDI-MS) at the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Once the identity of the peptides was found, they were individually synthesized by standard chemical methods. Using known animal and in vitro methods, the immunological function of these peptides was analyzed. The in vivo study of the effect of the peptides on immunity was performed using methods described in the following references. The peptides are given codes having the letters CMS followed by a number. The peptide sequence and the corresponding ID numbers are shown in Table A. A total of 30 peptides have been identified as having in vivo biological activities. The effect of the peptides on kidney function, liver function, cancer and body weight was also analyzed.

Table A

Sequence Listing	Peptide	Peptide Sequence

ID No.	Name	
1	CMS001	Pro Thr Thr Lys Thr Tyr Phe Pro His Phe
2	CMS002	Val Val Tyr Pro Trp Thr Gln Arg Phe
3	CMS008	Lys Ala Val Gly His Leu Asp Asp Leu Pro Gly Ala Leu
4	CMS010	Val Ala Pro Glu Glu His Pro Thr Leu Leu Thr Glu Ala Pro Leu Asn Pro Lys
5	CMS012	Leu Gly Met Glu Ala Cys Gly Ile His Glu Thr Thr Tyr
6	CMS013	Leu Arg Val Ala Pro Glu Glu His Pro Val Leu
7	CMS014	Ala Ala His His Pro Asp Asp Phe Asn Pro Ser Val
8	CMS015	Pro Ser Ile Val Gly Arg Pro Arg His Gln Gly Val Met
9	CMS016	Ile Gly Met Glu Ser Ala Gly Ile His Glu Thr Thr Tyr
10	CMS018	Val Gly Met Gly Glu Lys Asp Ser Tyr
11	CMS019	Val Gly Met Gly Gln Lys Asp Ser Tyr
12	CMS020	Val Gly Met Gly Gln Lys Asp Ser Tyr Val
13	CMS021	Met Ala Thr Ala Ala Ser Ser Ser Ser Leu
14	CMS022	Tyr Ser Phe
15	CMS023	Ala Ala Phe
16	CMS024	Tyr Ser Leu
17	CMS026	Thr Thr Tyr Asn Ser Ile Met
18	CMS027	Phe Glu Glu Asn Met
19	CMS028	Phe Glu Pro Ser Phe
20	CMS029	Phe Asn Glu Glu
21	CMS030	Phe Glu Glu Met
22	CMS032	Phe Glu Glu Glu
23	CMS033	Phe Glu Ser Phe
24	CMS034	Pro Glu Asn Phe
25	CMS035	Phe Val Asn Asp
26	CMS036	Phe Gln Pro Ser Phe
27	CMS003	Phe Asn Phe Val Pro Pro
28	CMS007	Ala Gly Asp Asp Ala Pro Arg Ala Val Phe
29	CMS009	Leu Arg Val Ala Pro Glu Glu His Pro Thr Leu
30	CMS011	Arg Val Ala Pro Glu Glu His Pro Thr Leu

One aspect of the present invention relates to substantially pure peptides having sequences identified as sequence ID No.1 to sequence ID No.30 operably linked to a molecule that enhances their therapeutic effectiveness. Candidate molecules to be operably linked to said peptides of SEQ ID NOs. 1-30 and the means for carrying out such linkings are familiar to those with skill in the art. Another aspect of the present invention relates to a substantially pure peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30, said peptide being operably linked to a molecule which enhances its therapeutic effectiveness. Some molecules that could be operably linked to said peptides of SEQ ID NOs. 1-30 include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The invention also relates to substantially pure peptides having sequences identified as sequence ID No.1 to sequence ID No.30 operably linked to a molecule that enhances its therapeutic effectiveness, wherein said operably linked molecule

is not a peptide which is adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. The molecule may be operably linked to the peptide of the invention with a covalent bond or a non-covalent interaction. In specific embodiments, the peptides can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight.

In specific embodiments, the biologically effective molecules, when operably linked to peptides of SEQ ID NOs. 1-30, can alter the pharmacokinetics of said peptides by conferring properties to the peptide as part of a linked molecule. Some of the properties that the operably linked molecules can confer on said peptides include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permeability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect. Said molecule which enhances said therapeutic effectiveness has an effect selected from the group consisting of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased intensity of effect, a decreased intensity of effect, a reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual.

Another aspect of the present invention relates to substantially pure peptides consisting essentially of sequences identified as sequence ID No.1 to sequence ID No.30 operably linked to a molecule which enhances its therapeutic effectiveness. In specific embodiments, the peptides above can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight. The invention also relates to substantially pure peptides consisting essentially of sequences selected from the list consisting of SEQ ID NOs. 1-30 operably linked to a molecule which enhances its therapeutic effectiveness, wherein said operably linked molecule is not a peptide which is adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. Some of the properties that the operably linked molecules can confer on said peptides of SEQ ID NOs. 1-30 include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permeability of a peptide; changing the bioavailability or the rate of delivery to the body of a

peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect. Said molecule which enhances said therapeutic effectiveness has an effect selected from the group consisting of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased intensity of effect, a decreased intensity of effect, a reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual. Some molecules that could be linked operably with said peptides of SEQ ID NOs. 1-30 include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The molecule may be operably linked to the peptide of the invention with a covalent bond or a non-covalent interaction.

Yet another aspect of the present invention relates to substantially pure peptides consisting of sequences identified as sequence ID No.1 to sequence ID No.30 operably linked to a molecule which enhances its therapeutic effectiveness. In specific embodiments, the peptides above can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight. The invention also relates to substantially pure peptides consisting essentially of sequences selected from the list consisting of SEQ ID NOs. 1-30 operably linked to a molecule which enhances its therapeutic effectiveness, wherein said operably linked molecule is not a peptide which is adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. Some of the properties that the operably linked molecules can confer on said peptides of SEQ ID NOs. 1-30 include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permeability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect. Said molecule which enhances said therapeutic effectiveness has an effect selected from the group consisting of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased intensity of effect, a decreased intensity of effect, a reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual.Some molecules that

could be linked operably with said peptides of SEQ ID NOs. 1-30 include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The molecule may be operably linked to the peptide of the invention with a covalent bond or a non-covalent interaction.

The invention also relates to substantially pure peptides comprising functional derivatives of peptides selected from the group consisting of SEQ ID NOs. 1-30 that are operably linked to a molecule which enhances its therapeutic effectiveness. Thus the present invention relates also to a substantially pure peptide consisting of an amino acid sequence which is a functional derivative of a biologically active peptide, this biologically active peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30, that is operably linked to a molecule that enhances its therapeutic effectiveness. It also relates to substantially pure peptides consisting of or consisting essentially of amino acid sequences which are functional derivatives of biologically active peptides, these biologically active peptides having amino acid sequences selected from the group consisting of SEQ ID NOs. 1-30, that are operably linked to a molecule that enhances its therapeutic effectiveness, wherein said operably linked molecule is not a peptide which is adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide In specific embodiments, the peptides above can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight. Some examples of biologically effective molecules that could be attached to the peptides described above include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The molecule may be operably linked to the peptide of the invention with a covalent bond or a non-covalent interaction. In specific embodiments, the operably linked biologically effective molecules can alter the pharmacokinetics of the peptides of the above described embodiments of the invention by virtue of conferring properties to the peptide as part of a linked molecule. Some of the properties that the operably linked molecules can confer on said peptides of SEQ ID NOs. 1-30 include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permeability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the

stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect. Said molecule which enhances said therapeutic effectiveness has an effect selected from the group consisting of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased intensity of effect, a decreased intensity of effect, a reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual.

Another aspect of the present invention relates to hybrid peptides containing a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 with an additional peptide sequence attached, where said attached additional sequence is not a sequence found adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. The present invention also relates to hybrid peptides containing a functional derivative of a biologically active peptide, this biologically active peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30, with an additional peptide sequence attached, wherein said attached additional sequence is not a sequence found adjacent to said peptide of SEQ ID NOs. 1-30 or said functional derivative in a naturally occurring peptide. In specific embodiments, the hybrid peptides above can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight. In specific embodiments, these attached additional peptide sequences not found adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide, can alter the pharmacokinetics of the peptides of the above described embodiments of the invention by virtue of conferring properties to the peptide as part of a hybrid molecule. Some of the properties that the operably linked molecules can confer on said peptides of SEQ ID NOs. 1-30 include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permeability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect.

Another aspect of the present invention relates to a genetic vector comprising a first nucleotide sequence encoding a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 fused in frame with a second nucleotide sequence encoding a peptide that enhances the therapeutic effectiveness of the aforementioned peptide and that is not adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. It also relates to a genetic vector comprising a first nucleotide sequence encoding a peptide consisting essentially of an amino acid sequence selected from the group consisting of SEQ

ID NOs. 1-30 fused in frame with a second nucleotide sequence encoding a peptide that enhances the therapeutic effectiveness of the aforementioned peptide and that is not adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. It further relates to a genetic vector comprising a first nucleotide sequence encoding a peptide consisting of an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 fused in frame with a second nucleotide sequence encoding a peptide that enhances the therapeutic effectiveness of the aforementioned peptide and that is not adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. The invention also relates to a genetic vector comprising a nucleotide sequence encoding a peptide comprising a functional derivative of a biologically active amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 and further comprising an additional adjacent nucleotide sequence encoding a peptide that is not a peptide sequence found adjacent to said peptide of SEQ ID NOs. 1-30 or said functional derivative in a naturally occurring peptide. It also relates to a genetic vector comprising a nucleotide sequence encoding a peptide consisting essentially of a functional amino acid sequence which is a functional derivative of a biologically active amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 and further comprising an additional adjacent nucleotide sequence encoding a peptide that is not a peptide sequence found adjacent to said peptide of SEQ ID NOs. 1-30 or said functional derivative in a naturally occurring peptide. It also relates to a genetic vector comprising a nucleotide sequence encoding a peptide consisting of a functional amino acid sequence which is a functional derivative of a biologically active amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 and further comprising an additional adjacent nucleotide sequence encoding a peptide that is not a peptide sequence found adjacent to said peptide of SEQ ID NOs. 1-30 or said functional derivative in a naturally occurring peptide. In specific embodiments, said peptides selected from the list consisting of SEQ ID NOs. 1-30 can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight. Some of the properties that the operably linked molecules can confer on said peptides of SEQ ID NOs. 1-30 include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permeability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect. Said molecule which enhances said therapeutic effectiveness has an effect selected from the group consisting of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased intensity of effect, a decreased intensity of effect, a

reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual. Another aspect of the invention relates to micro-organisms that comprise nucleic acid sequences selected from the list consisting of: the nucleotide sequences of the vectors described above; a nucleotide sequence comprising a first nucleotide sequence encoding a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 fused in frame with a second nucleotide sequence encoding a peptide that is not adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide; and a nucleotide sequences comprising a first nucleotide sequence encoding a peptide consisting of a functional amino acid sequence which is a functional derivative of a biologically active amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 and fused in frame with a second nucleotide sequence encoding a peptide that is not a peptide sequence found adjacent to said peptide of SEQ ID NOs. 1-30 or said functional derivatives in a naturally occurring peptide.

In a specific embodiment, the hybrid peptides produced in any of the above-described genetic vectors can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight.

An additional aspect of the present invention relates to a method of enhancing the therapeutic effect of a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 comprising operably linking said peptide to a molecule which enhances said therapeutic effect. The invention also relates to said method wherein said peptide can modulate, but is not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight. Some examples of biologically effective molecules that could be attached to said peptides of SEQ ID NOs. 1-30 include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The invention also relates to a method of enhancing the therapeutic effect of a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 comprising operably linking said peptide to a molecule which enhances said therapeutic effect, wherein said method which enhances said therapeutic effect is not the inclusion of a peptide which is adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide, and wherein said molecule is not a peptide which is adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. The molecule may be operably linked to the peptide of the

invention with a covalent bond or a non-covalent interaction. In a specific embodiment, the properties that said linked molecule can confer on said peptides to enhance their therapeutic effects include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permeability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect. Said molecule which enhances said therapeutic effectiveness has an effect selected from the group consisting of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased intensity of effect, a decreased intensity of effect, a reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual.

Yet another aspect of the present invention relates to a pharmaceutical composition comprising a substantially pure peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 operably linked to a molecule which enhances its therapeutic effectiveness. The invention also relates to pharmaceutical composition comprising a substantially pure peptide consisting of or consisting essentially of an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 operably linked to a molecule which enhances its therapeutic effectiveness. In specific embodiments, the pharmaceutical compositions above can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight. The invention also relates to pharmaceutical compositions comprising peptides comprising sequences selected from the list consisting of SEQ ID NOs. 1-30 operably linked to a molecule which enhances its therapeutic effectiveness, wherein said operably linked molecule is not a peptide which is adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. Some of the properties that the operably linked molecules can confer on said peptides of SEQ ID NOs. 1-30 include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permeability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect. Said molecule which enhances said therapeutic effectiveness has an effect selected from the group consisting of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased

intensity of effect, a decreased intensity of effect, a reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual.Some molecules that could be linked operably with said peptides of SEQ ID NOs. 1-30 include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The molecule may be operably linked to the peptide of SEQ ID NOs. 1-30 with a covalent bond or a non-covalent interaction.

The present invention also relates to a pharmaceutical composition comprising a substantially pure peptide comprising a functional derivative of a biologically active peptide, this biologically active peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30, operably linked to a molecule that enhances its therapeutic effectiveness. It also relates to a pharmaceutical composition comprising a substantially pure peptide consisting essentially of a functional derivative of a biologically active peptide, this biologically active peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30, operably linked to a molecule that enhances its therapeutic effectiveness. It further relates to pharmaceutical composition comprising a substantially pure peptide consisting of functional derivative of a biologically active peptide, this biologically active peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30, operably linked to a molecule that enhances its therapeutic effectiveness. In specific embodiments, the pharmaceutical compositions above can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight. The invention also relates to pharmaceutical compositions comprising a functional derivative of a biologically active peptide, this biologically active peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30, operably linked to a molecule that enhances its therapeutic effectiveness, wherein said operably linked molecule is not a peptide which is adjacent to said peptide of SEQ ID NOs. 1-30 or said functional derivative in a naturally occurring peptide. Some of the properties that the operably linked molecules can confer on said functional derivatives of biologically active peptides of SEQ ID NOs. 1-30 include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permeability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of

the effects of a peptide; providing a permissive action by allowing a peptide to have an effect. Said molecule which enhances said therapeutic effectiveness has an effect selected from the group consisting of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased intensity of effect, a decreased intensity of effect, a reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual.Some molecules that could be linked operably with said functional derivatives of biologically active peptides of SEQ ID NOs. 1-30 include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The molecule may be operably linked to the functional derivative of a peptide of SEQ ID NOs. 1-30 with a covalent bond or a non-covalent interaction.

In a specific embodiment, the peptides present in any of the above-described pharmaceutical compositions can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight.

A further aspect of the present invention relates to a method of making a pharmaceutical composition comprising providing a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 operably linked to a molecule which enhances its therapeutic effect; and formulating said peptide operably linked with said molecule with a pharmaceutically acceptable carrier. It further relates to a method of making a pharmaceutical composition comprising obtaining a peptide comprising, consisting of, or consisting essentially of an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 operably linked to a molecule which enhances its therapeutic effect; and formulating said peptide operably linked with said molecule with a pharmaceutically acceptable carrier.The invention also relates to said method wherein said peptide can modulate, but is not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight. Some examples of biologically effective molecules that could be attached to said peptides of SEQ ID NOs. 1-30 include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The invention also relates to a method of making of pharmaceutical comprising a peptide comprising an amino

acid sequence selected from the group consisting of SEQ ID NOs. 1-30 comprising operably linking said peptide to a molecule which enhances said therapeutic effect, wherein said molecule is not a peptide which is adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. The molecule may be operably linked to the peptide of the invention with a covalent bond or a non-covalent interaction. In a specific embodiment, the properties that said linked molecule can confer on said peptides to enhance their therapeutic effects include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permeability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect. Said molecule which enhances said therapeutic effectiveness has an effect selected from the group consisting of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased intensity of effect, a decreased intensity of effect, a reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual. It also relates to a method of making a pharmaceutical composition comprising providing a substantially pure peptide consisting essentially of an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 operably linked to a molecule which enhances its therapeutic effect; and formulating said peptide operably linked with said molecule with a pharmaceutically acceptable carrier. It further relates to a method of making a pharmaceutical composition comprising providing a substantially pure peptide consisting of or consisting essentially of an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 operably linked to a molecule which enhances its therapeutic effect; and formulating said peptide operably linked with said molecule with a pharmaceutically acceptable carrier.

Another aspect of the present invention is a method of making a pharmaceutical composition comprising providing a substantially pure peptide comprising an amino acid sequence which is a functional derivative of a biologically active peptide, this biologically active peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30, operably linked to a molecule which enhances its therapeutic effect; and formulating said peptide operably linked with said molecule with a pharmaceutically acceptable carrier.

It further relates to a method of making a pharmaceutical composition comprising providing a substantially pure peptide consisting essentially of an amino acid sequence which is a functional derivative of a biologically active peptide, this biologically active peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30, operably linked to a molecule which enhances its therapeutic effect; and formulating

said peptide operably linked with said molecule with a pharmaceutically acceptable carrier. It also relates to a method of making a pharmaceutical composition comprising providing a substantially pure peptide consisting of an amino acid sequence which is a functional derivative of a biologically active peptide, this biologically active peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30, operably linked to a molecule which enhances its therapeutic effect; and formulating said peptide operably linked with said molecule with a pharmaceutically acceptable carrier.

In connection with any of the above-described method, the peptide can modulate, but not limited to modulating, one or more of the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight.

Yet a further aspect of the present invention relates to a method of treatment of a human comprising administering a pharmaceutically effective dose of a substantially pure peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 to a human, said peptide being operably linked to a molecule which enhances its therapeutic effectiveness. It also relates to a method of treatment of a human comprising administering a pharmaceutically effective dose of a substantially pure peptide comprising an amino acid sequence which is a functional derivative of a biologically active peptide, this biologically active peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30. The present invention also relates to a method of treatment of a human comprising administering a pharmaceutically effective dose of a substantially pure peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1-30 to a human, said method which enhances said therapeutic effectiveness is not a peptide which is adjacent to said peptide of SEQ ID NOs. 1-30 in a naturally occurring peptide. Said molecule which enhances said therapeutic effectiveness has an effect selected from the group consisting of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased intensity of effect, a decreased intensity of effect, a reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual. Some molecules that could be operably linked to said peptides of SEQ ID NOs. 1-30 include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The molecule may be operably linked to the peptide of the invention with a covalent bond or a non-covalent interaction.In a specific embodiment, the peptides used for the treatment of human described above may be used to modulate, but not limited to modulating, one or more of the following human conditions: immune activity; hepatitis

infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight.

In connection with any of the above-described nucleic acid sequences, the peptides and/or hybrid peptides expressed from these nucleic acid sequences can modulate, but not limited to modulating, the following: immune activity; hepatitis infection, including but not limited to hepatitis B infection; nephritis; the growth of a cancer, including but not limited to sarcoma, liver cancer, leukemia and melanoma; and body weight.

As used herein, the terminology "consisting essentially of" refers to a peptide or polypeptide which includes the amino acid sequence of the peptides selected from the list consisting of SEQ ID NOs. 1-30 along with additional amino acids at the carboxyl and/or amino terminal ends and which maintains the activity of said peptides provided herein. Thus, as a non-limiting example, where the activity of the peptide selected from the list consisting of SEQ ID NOs. 1-30 is to modulate immune activity, a peptide or polypeptide "consisting essentially of" the peptide selected from the list consisting of SEQ ID NOs. 1-30 will possess the activity of modulating immune activity as provided herein with respect to that peptide and will not possess any characteristics in and of itself (i.e. before modification by attachment to one or more biologically active molecules) which materially reduces the ability of the peptide or polypeptide to modulate immune activity or which constitutes a material change to the basic and novel characteristics of the peptide as a modulator of immune activity. Thus, in the foregoing example, a full length naturally occurring polypeptide which has a primary activity other than modulating immune activity and which contains the amino acid sequence of a peptide selected from the list consisting of SEQ ID NOs. 1-30 somewhere therein would not constitute a peptide or polypeptide "consisting essentially of" a peptide selected from the list consisting of SEQ ID NOs. 1-30.. Likewise, in the foregoing example, a genetically engineered peptide or polypeptide which has a primary activity other than modulating immune activity but includes the amino acid sequence of a peptide selected from the list consisting of SEQ ID NOs. 1-30 somewhere therein would not constitute a peptide or polypeptide "consisting essentially of" a peptide selected from the list consisting of SEQ ID NOs. 1-30..

Besides the example of immune activity modulation used for illustration above, the foregoing definition also applies to all the peptides selected from the list consisting of SEQ ID NOs. 1-30 with respect to the activities provided for such peptides before operably linking said peptides to a molecule that enhances their therapeutic effectiveness. In particular, the foregoing definition applies to peptides selected from the list consisting of SEQ ID NOs. 1-30 having activities in modulating the extent of a viral infection, modulating the extent of a hepatitis infection, modulating the extent of nephritis, modulating the growth of a cancer, or modulating body weight as set forth in the detailed description below.

Those skilled in the art can readily determine whether a peptide or polypeptide consists essentially of a peptide selected from the list consisting of SEQ ID NOs. 1-30 under

the foregoing definitions by measuring the activity of the peptide or polypeptide using the assays for modulation of immune activity, modulating the extent of a viral infection, modulating the extent of a hepatitis infection, modulating the extent of nephritis, modulating the growth of a cancer, or modulating body weight which are provided herein with respect to a particular peptide selected from the list consisting of SEQ ID NOs. 1-30.

In the preferred embodiment, the terminology "consisting essentially of" may also refer to peptides or polypeptides which have less than 20 amino acid residues in addition to the peptide selected from the list consisting of SEQ ID NOs. 1-30. In a more preferred embodiment, the same terminology refers to a peptides with less than 15 amino acid residues in addition to the selected from the list consisting of SEQ ID NOs. 1-30. In an even more preferred embodiment, the same terminology refers to a peptides with less than 10 amino acid residues in addition to the peptide selected from the list consisting of SEQ ID NOs. 1-30. In another preferred embodiment, the same terminology refers to peptides or polypeptides with less than 6 amino acids in addition to one of the peptide selected from the list consisting of SEQ ID NOs. 1-30. In another preferred embodiment, the same terminology refers to peptides or polypeptides with less than 4 amino acids in addition to one of the peptide selected from the list consisting of SEQ ID NOs. 1-30. In the most preferred embodiment, the same terminology refers to peptides or polypeptides with less than 2 amino acids in addition to one of the peptide selected from the list consisting of SEQ ID NOs. 1-30.

BRIEF DESCRIPTION OF THE DRAWINGS

Each of the five figures demonstrates exemplary chemical reactions for linking peptides tc steroid molecules.

Figure 1 shows a series of chemical reactions for linking a peptide to an estrone molecule with a covalent bond.

Figure 2 shows a second, alternative set of reactions for creating the same linkage as in Figure 1.

Figure 3 contains a series of chemical reactions designed to link a peptide to a molecule of estradiol with a covalent bond.

Figure 4 contains a second series of chemical reactions for creating the same linkage as in Figure 3.

Figure 5 demonstrates a method of linking a peptide via a covalent bond to a molecule of hydrocortisone.

DETAILED DESCRIPTION OF THE INVENTION

The peptides can be readily synthesized by standard synthetic methods from L-amino acids, but may also be synthesized by genetic engineering methods using nucleic acids that have sequences encoding the individual peptides.

I. BIOLOGICAL ACTIVITY

In order to investigate the possible biological activity of peptides, the immunological effect of the peptides on animal model was examined, with procedures compliant to the "Principles of Pre-clinical Research of New Drugs" issued by Ministry of Health of People's Republic of China [1].

The T lymphocyte transformation test, NK cell cytotoxicity activity test, and the T lymphocyte IL-2 and IFN-γ secretion test were used to detect any possible effect of peptides on specific cellular immune function. The carbon particle clearance test was used to detect any possible effect of peptides on non-specific cellular immune function. The Sheep Red Blood Cell (SRBC) hemolysis test was used to detect any possible effect of peptides on humoral immune function. The immunity organ weight test was used to detect any possible effect of peptides at the organ level.

In this study, the saline group was used as negative control, while the IL-2 and IFN-γ groups were used as positive controls, since IL-2 and IFN-γ are well-studied immunostimulants [10]. Four arbitrary concentrations of sample peptides were used in this study, to cover a 1000 fold dosage range. Due to the intrinsic complexity of in vivo immunological response, and the lack of prior knowledge on the dosage versus response function, therefore any statistically significant difference over the negative control in any of the dosage groups has been scored as positive biological activity.

Results of this study were as follows:

1. Peptides CMS001, CMS002, CMS003, CMS007, CMS008, CMS009, CMS010, CMS011, CMS012, CMS015, CMS019, CMS021, CMS029, and CMS034 were found to be able to enhance T lymphocyte transformation, having statistically significant difference from the saline normal control group. Peptides CMS014 and CMS036 were also found to be able to inhibit T lymphocyte transformation, having statistically significant difference from the saline normal control group.

2. Peptides CMS001, CMS002, CMS003, CMS008, CMS009, CMS010, CMS011, CMS012, CMS013, CMS015, CMS016, CMS020, CMS021, CMS022, CMS023, CMS024, CMS026, CMS027, CMS028, CMS029, CMS030, CMS032, CMS033, CMS034, CMS035, and CMS036 were found to be able to increase the cytotoxic activity of NK cells, having statistically significant difference from the saline normal control group. Peptides CMS008 and CMS012, at suitable concentration, were also found to be able to decrease the cytotoxic activity of NK cells, having statistically significant difference from the saline normal control group.

3. Peptides CMS001, CMS003, CMS007, CMS009, CMS010, CMS011, CMS012, CMS015, CMS020, CMS022, and CMS034 were found to be able to enhance the secretion of interleukin-2 (IL-2) by T lymphocytes, having statistically significant difference from the saline normal control group.

4. Peptides CMS001, CMS003, CMS009, CMS010, CMS011, CMS012, CMS013, CMS016, CMS021, CMS022, and CMS028 were found to be able to enhance the secretion of IFN by T lymphocytes, having statistically significant difference form the saline normal control group.

5. Peptides CMS001, CMS002, CMS003, CMS007, CMS008, CMS009, CMS010, CMS011, CMS012, CMS013, CMS014, CMS015, CMS016, CMS018, CMS019, CMS020, CMS021, CMS022, CMS023, CMS024, CMS026, CMS027, CMS028, CMS029, CMS030, CMS032, CMS033, CMS034, CMS035, and CMS036 were found to be able to enhance the synthesis of anti-SRBC antibody upon the antigenic challenge, having statistically significant difference from the saline normal control group. Peptides CMS002, CMS003, CMS009, CMS010, CMS011, CMS013, CMS014, CMS015, CMS018, CMS019, CMS020, CMS026, CMS028, CMS029, CMS030, CMS034, and CMS036, at suitable concentration, were also found to be able to inhibit the synthesis of anti-SRBC antibody upon the antigenic challenge, having statistically significant difference from the saline normal control group.

6. Peptides CMS003, CMS008, CMS009, CMS010, CMS011, CMS013, CMS016, CMS018, CMS019, CMS020, CMS022, CMS024, CMS027, CMS030, CMS035, CMS036 were found to be able to enhance the phagocytotic activity of mononuclear phagocyte, having statistic significant difference from the saline normal control group.

7. Peptides CMS001, CMS002, CMS008, CMS010, CMS012, CMS013, CMS014, CMS015, CMS016, CMS018, CMS019, CMS020, CMS021, CMS022, CMS023, CMS024, CMS026, CMS027, CMS028, CMS029, CMS030, CMS032, CMS033, CMS034, CMS035, and CMS036 were found to be able to increase the weight of the thymus gland, having statistically significant difference from the saline normal control group.

8. Peptides CMS019, CMS020, and CMS030 were found to be able to increase the weight of the spleen, having statistically significant difference from the saline normal control group. Peptides CMS001, CMS003, CMS007, CMS008, CMS009, CMS010, CMS011, CMS013, CMS014, CMS015, CMS021, CMS023, CMS024, CMS027, CMS029, and CMS036, at suitable concentration, were also found to be able to decrease the weight of the spleen, having statistically significant difference from the saline normal control group.

The materials and methods used to analyze the effect of the peptides on mouse are described below.

Materials

1. Experimental Animal

BALB/c Mice, 18-22g weight, 50% female and 50% male, provided by Experimental Animal Center, National Institute of Medical Science, PR China.

2. Administration

recombinant mouse IFN-γ (rmIFN-γ) group: 3×10^5IU/kg/day
recombinant human IL (rhIL)-2 group: 3×10^5IU/kg/day
Saline group: 0.5ml/each/day
peptide dose I group: 500μg/kg/day
peptide dose II group: 50μg/kg/day
peptide dose III group: 5μg/kg/day
peptide dose IV group: 0.5μg/kg/day

The above substances were all dissolved in 0.5ml saline and injected intraperitoneal (i.p.) for 15 continuous days, once per day.

3. Main Reagents

The peptides were custom manufactured by American Peptide Company, Inc., USA
Fetal bovine serum, and RPMI-1640 cell culture medium, Gibco, USA
MTT, and ConA, Sigma, USA
rmIFN-γ, Beijing Biotech Inc., China
rhIL-2, Shanghai Huaxin Biotech Inc., China
Lymphocyte separation solution, Research Institute of Hematologic Disease, National Institute of Medical Science, PR China
Vesicular Stomatitis Virus (VSV), IFN-γ and IL-2 standard sample, National Institute For The Control Of Pharmaceutical And Biological Products, PR China
HT-2 cell and L929 cell, gift from Prof. WF Chen of Beijing University Department of Immunology, PR China

METHOD

1. The effect of peptides on cellular immunity

1.1 Preparation of spleen cell suspension [1,2]

The BALB/c mice were randomly divided into the peptide, IFN, IL-2, and saline groups. Ten mice per group. The day after the last test substance administration, the mice were sacrificed by cervical dislocation. The spleen was isolated aseptically and manually dispersed in cold D-Hank's solution using an injection needle. The dispersed cell suspension

was further sieved through a 100 gauge 150μm diameter stainless steel sieve. After centrifugation at 200g for 10 minutes, the supernatant was discarded. The cell pellet was re-suspended in 10 volume of Tris-NH_4Cl buffer and then kept standing still for 10 minutes at room temperature. The suspended cells were collected by centrifugation at 150g for 10 minutes. The cells were washed 2-4 times with cold D-Hank's solution by re-suspending and collecting by centrifugation with condition as described above. The washed cells were then diluted to the desired cell densities by RPMI-1640 culture medium, containing 10% fetal bovine serum.

1.2 The effect of peptides on T lymphocyte transformation[1, 2]

Spleen cells of density 1×10^6/ml were placed onto a 96 wells cell culture plate, 100μl/well, three parallel wells each of the assay sample and control sample per mouse. To the assay wells, 100μl/well ConA of 100μg/ml in RPMI-1640 was added, and 100μl/well plain RPMI-1640 was used for the controls. The cells were incubated for 66 hrs at 37°C, 5% CO_2. The cells were then pelleted by centrifugation at 150g for 10 minutes. The supernatant was collected and stored at –20°C for cytokines IL-2 and IFN determination.

50μl/well MTT of 1mg/ml in RPMI-1640 was added to the cell pellet and the cells re-suspended by shaking for 2 minutes. The incubation was continued for 4 hours. The supernatant was discarded after centrifugation at 150g for 10 minutes. 120μl 40mM HCl-2-propanol was added to the cell pellet and shaken for 3 minutes. to obtain OD_{570}nm of each well referenced at 630nm. An ELISA reader was used

Calculation:

Each mouse formed three assay wells and three control wells. The Stimulation Index (SI) of each mouse was obtained by first deriving the average OD of the three parallel wells, then dividing the value of the assay wells by the control wells.

1.3 The effect of peptides on NK cell activity[3, 4]

Mice spleen cells were prepared to $4\text{x}10^6$/ml as described in section 1.1 above. Target cells YAC-1 were brought to log phase and adjusted to $1\text{x}10^5$/ml. Using a 96 wells cell culture plate, 100μl mouse spleen cells and 100μl culture medium were added to the control well containing only the spleen cells; 100μl target cells and 100μl culture medium were added to the control well containing_only target cells; 100μl mouse spleen cells and 100μl target cells were added to the NK activity assay well. Three parallel sets of the above were prepared per mouse.

Samples were centrifuged at 150g for 10 minutes to collect the cells. The supernatant was discarded and 50μl/well MTT of 1mg/ml was added. The reaction_mixture was then shaken for 2 minutes, and incubated at 37°C, 5% CO_2 for 4 hours. The supernatant was discarded after centrifugation at 150g for 10 minutes. 120 μl 40mM HCl-2-propanol

was added and shaken for 3 minutes. An ELISA reader was used to obtain OD_{570nm} of each well referenced at 630nm.

Calculation:

Each mouse has 9 wells: three spleen cells only control, three target cells only control, and three assay wells with both spleen and target cells. The NK cell activity index of each mouse was obtained by first deriving the average OD of the three parallel wells of each combination, then applying this average OD to the following formula:

NK cell activity index = [1-(average OD of spleen and target cell well – average OD of spleen cell only well) ÷ (average OD of target cell only well)] x 100%

1.4 The effect of peptides on the activity T lymphocyte in secreting IL-2[6]

HT-2 cells at log phase were collected by centrifugation at 150g for 10 minutes, and washed three times with cold Hank's solution by re-suspension and centrifugation. The collected HT-2 cells were re-suspended in RPMI-1640 and incubated at 37°C, 5% CO_2 for 30 minutes. The cells were further washed twice with RPMI-1640 by re-suspension and centrifugation, and re-suspended to final concentration of 2×10^5/ml with RPMI-1640.

The supernatant obtained in section 1.2 were diluted to the following percentage with RPMI-1640: 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%.

rIL-2 was diluted to the following concentration with RPMI-1640: 500IU/ml, 250IU/ml, 125IU/ml, 62.5IU/ml, 31.25IU/ml, and 15.5IU/ml.

A 96 well cell culture plate was set up with three parallel wells per combination:

Negative control: 100µl RPMI-1640 + 100µl HT-2 cell suspension

rIL-2 standard: 100µl rIL-2 solution + 100µl HT-2 cell suspension

Assay well: 100µl diluted supernatant + 100µl HT-2 cell suspension

The plate was incubated at 37°C, 5% CO_2 for 68 hours, then centrifuged at 150g for 15 minutes and the supernatant removed. 100µl 0.5mg/ml MTT in RPMI-1640 without phenolsulfonphthalein was added into each well. After shaking for 3-4 minutes to re-suspend the cells, continue to incubate for another 4 hours. Samples were then centrifuged at 150g for 15 minutes and the supernatant was removed. To each well, 120µl 40mM HCl-2-propanol was added, mixed for 3-4 minutes and OD analyzed at 570nm, referenced at 630nm with an ELISA plate reader.

Calculation:

The average OD of the three parallel wells of each dilution was taken and plotted against concentration on a semi-log paper, concentration on the X-axis. The concentration at 50% OD saturation was obtained for both the testing supernatant and rIL-2.

Sample IL-2 activity = (sample dilution at 50% maximum action ÷ rIL-2 standard dilution at 50% maximum action) x activity of rIL-2 standard at 50% maximum action (IU/ml)

1.5 The effect of peptides on the activity of T lymphocyte in secreting interferon (IFN)[6]

The supernatant from the section 1.2 was diluted with RPMI-1640 culture medium to the following percentages: 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%.

The recombinant interferon (rIFN) standard was diluted with RPMI-1640 to the following concentrations: 500IU/ml, 250IU/ml, 125IU/ml, 62.5IU/ml, 31.25IU/ml, and 15.5IU/ml.

Target cells L929 at log phase were adjusted to $2x10^5$/ml with RPMI-1640, with treatment same as the HT-2 cell described in section 1.4. Stock VSV was also adjusted to 100 $TCID_{50}$ with RPMI-1640.

The following on a 96 well culture plate was set up, three parallel wells per combination:

Negative control well: 150μl RPMI-1640 + 100μl L929

Positive control well: 100μl RPMI-1640 + 100μl L929 + 50μl VSV

rIFN activity well: 100μl rIFN standard + 100μl L929 + 50μl VSV

assay well: 100μl diluted supernatant + 100μl L929 + 50μl VSV

Samples were incubated at 37°C, 5% CO_2 for 24 hours. The positive control wells were observed periodically under inverted microscope to confirm cell lysis, then collected, washed, and OD of all wells was read same as described in section 1.4.

Calculation:

Concentrations at 50% maximum action were obtained same way as section 1.4. Calculate IFN activity of sample as following:

Sample IFN activity = (sample dilution at 50% maximum action ÷ rIFN standard dilution at 50% maximum action) x standard rIFN activity at that 50% maximum action (IU/ml)

2. The effect of peptides on antibody formation[7]

Sheep red blood cells (SRBC) were prepared by collecting blood from cervical vein and put into a sterile flask with glass beads. The flask was shaken for 3 minutes and the blood then mixed with Alsever solution (glucose 2.05g, NaCl 0.4g, Na lemonade 0.8g, adjust to 100ml with distilled water) and stored at 4°C. Immediately before use, samples were centrifuged at 130g, 5 minutes to collect the SRBC. The cells were washed two times by re-suspension and centrifugation in normal saline. Then the cell pellet was collected by centrifugation at 180g for 10 minutes and re-suspended in saline to make the final working SRBC suspension, 2% (v/v).

Complement was prepared by adding 10 volumes of fresh Cavy serum into one volume centrifuge packed SRBC, and then gently shaking for 30 minutes at 4°C. The SRBC was removed by centrifugation at 200g for 10 minutes. 10 volumes of normal saline were added to obtain the working complement solution.

The BALB/c mice were randomly divided into the peptide group, IFN group, IL-2 group, and saline group, 10 mice per group. The test substances were administered as described in section 1.1, plus intraperitoneal injection 0.2ml SRBC per mouse on day 12. On the day after the last test substance administration (day 16), blood was collected from the eye canthus and left at room temperature for one hour for serum exudation. After centrifugation at 200g for 10 minutes, the serum was diluted by 500 times with normal saline.

To 1ml diluted mouse serum of each mouse, 0.5ml SRBC suspension was added. Ice cold. Then 1ml working complement solution was added and incubated at 37°C water bath for 10 minutes. Reactions were terminated by ice cold. Samples were then centrifuged at 200g for 10 minutes to obtain the supernatant.

To 1ml of this supernatant, 3ml Drabkin solution was added and left at room temperature for 10 minutes. OD_{540nm} was obtained.

Calculation:

Reference OD_{540nm} was obtained by mixing 0.25ml SRBC suspension with Drabkin solution to 4ml and placed at room temperature for 10 minutes before OD_{540nm} was taken.

Sample serum index = (OD_{540nm} of test sample ÷ reference OD_{540nm}) x 500

3. The effect of peptides on the phagocytosis function of mononuclear phagocyte and the weight of immune organ[8, 9].

On the next day after the last test substance administration (day 16), the mice were injected with 0.1ml/kg body weight India ink (5 times dilution with normal saline) from the tail vein.

One minute and five minutes after Indian ink injection, 20μl blood was obtained from the eye canthus with a heparinized tubing. The blood was mixed with 2ml 0.1% w/v Na_2CO_3 and then OD_{680nm} obtained. The outline clear index K was calculated by the following formula:

$K = (\lg A_1 - \lg A_2) \div (t2 - t1)$

Key:

A1: OD680nm at first minute

A2: OD680nm at fifth minute

t2: 5 minutes

t2: 1 minute

One day after the last test substance administration (day 16), the liver, spleen, and thymus gland were separated and blotted dry with filter paper and weighed. The phagocytosis index α was calculated as below:

$\alpha = (\sqrt[3]{K})(W \div W_{LS})$

key:

W: body weight

W_{LS}: weight of liver plus spleen

Thymus gland index (%) = (thymus weight / body weight) × 100%

Spleen index (%) = (spleen weight / body weight) × 100%

Results

Due to the large quantity of raw data involved, only the compiled end result are presented. The groups without statistically significant difference from the saline negative control are also omitted.

1. The effect of peptides on T lymphocyte transformation

At 500μg/kg/day, CMS002, CMS007, CMS008, CMS009, CMS010, CMS012, CMS015, CMS019, CMS021, and CMS029 were found to be able to enhance T lymphocyte transformation, having statistically significant difference from the saline group ($P<0.05$). Among these peptides, CMS010 and CMS015 were found to have statistically significant difference from the IFN-γ group and IL-2 group ($P<0.05$) as shown in Table 1 below.

Table 1

Group	N	X±SD (stimulation index)
CMS002	8	1.8±0.3*
CMS007	9	1.6±0.1*
CMS008	9	1.7±0.1*
CMS009	10	1.7±0.2*
CMS010	9	2.0±0.3*@^
CMS012	9	1.6±0.2*
CMS015	9	1.9±0.3*@^
CMS019	9	1.8±0.3*
CMS021	10	1.6±0.1*
CMS029	9	1.7±0.3*
IFN-γ	10	1.6±0.2*
IL-2	10	1.7±0.2*
Saline	10	1.3±0.1

* comparing to saline group $P<0.05$

@ comparing to IFN-γ group $P<0.05$

^ comparing to IL-2 group $P<0.05$

At 50μg/kg/day, CMS001, CMS002 and CMS003 were found to be able to stimulate T lymphocyte transformation, having statistically significant difference from saline group, IFN-γ group, and IL-2 group ($P<0.05$). CMS014 and CMS036 were found to be able to inhibit T lymphocyte transformation, having statistical significant difference from saline group ($P<0.05$). Data detailed in Table 2 below.

Table 2

Group	N	X±SD (stimulation index)

CMS001	10	2.2±0.5*@^
CMS002	10	2.6±0.3*@^
CMS003	8	2.2±0.5*@^
CMS014	9	1.0±0.1*
CMS036	9	1.0±0.1*
IFN-γ	9	1.7±0.2*
IL-2	10	1.8±0.2*
Saline	10	1.3±0.1

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 5μg/kg/day, CMS001, CMS003, CMS007, and CMS034 were found to be able to stimulate T lymphocyte transformation, having statistically significant difference from the saline group (P<0.05) as shown in Table 3.

Table 3

Group	N	X±SD (stimulation index)
CMS001	10	1.7±0.2*
CMS003	10	1.6±0.2*
CMS007	8	1.7±0.1*
CMS034	9	1.5±0.2*
IFN-γ	10	1.6±0.2*
IL-2	9	1.6±0.1*
Saline	10	1.3±0.1

*comparing to saline group P<0.05

At 0.5μg/kg/day, CMS008, CMS010, and CMS011 were found to be able to stimulate T lymphocyte transformation, having statistically significant difference from the saline group (P<0.05) as shown in Table 4.

Table 4

Group	N	X±SD (stimulation index)
CMS008	10	1.7±0.3*
CMS010	9	1.7±0.3*
CMS011	10	1.6±0.4*
IFN-γ	10	1.6±0.2*
IL-2	10	1.6±0.1*
Saline	10	1.3±0.1

* comparing to saline group P<0.05

2. The effect of peptide on NK cell cytotoxic activity

At 500μg/kg/day, CMS010, CMS013, CMS016, CMS023, CMS024, CMS026, CMS027, CMS028, CMS029, CMS030, CMS032, CMS033, CMS034, CMS035, and CMS036 were found to be able to increase NK cell cytotoxic activity, with statistically

significant difference from the saline group (P<0.05). Among these peptides, CMS010, CMS016, and CMS030 were found to have statistically significant difference from the IFN-γ group and IL-2 group (P<0.05) as shown in Table 5.

Table 5

Group	N	X±SD (%)
CMS010	9	91±4*@^
CMS013	8	84±9*
CMS016	9	91±7*@^
CMS023	10	79±12*
CMS024	10	89±8*
CMS026	10	89±7*
CMS027	10	88±8*
CMS028	10	90±5*
CMS029	10	87±4*
CMS030	10	91±5*@^
CMS032	10	87±5*
CMS033	9	89±8*
CMS034	11	85±9*
CMS035	8	90±10*
CMS036	10	88±7*
IFN-γ	10	77±8*
IL-2	10	77±8*
Saline	8	63 ±9

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 50μg/kg/day, CMS001, CMS003, CMS015, CMS021, CMS026, and CMS035 were found to be able to increase NK cell cytotoxic activity, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS021 was found to have statistically significant difference from the IFN-γ group and IL-2 group (P<0.05). CMS012 was found to be able to inhibit NK cell cytotoxic activity, having statistically significant difference from the saline group (P<0.05). Data detailed in Table 6 below.

Table 6

Group	N	X±SD (%)
CMS001	10	85±10*
CMS003	10	85±6*
CMS012	9	40±9*
CMS015	8	78±8*
CMS021	8	88±12*@^
CMS026	10	76±9*
CMS035	10	72±9*
IFN-γ	10	73±10*
IL-2	10	74±8*
Saline	10	56±8

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 5μg/kg/day, CMS008, CMS009, CMS010, CMS011, CMS012, CMS020, CMS024, CMS034, and CMS036 were found to be able to increase NK cell cytotoxic activity, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS008 and CMS009 were found to have statistically significant difference from the IFN-γ group and IL-2 group (P<0.05) as shown in Table 7.

Table 7

Group	N	X±SD (%)
CMS008	10	92±4*@^
CMS009	8	92±6*@^
CMS010	10	82±9*
CMS011	10	76±10*
CMS012	10	85±7*
CMS020	9	91±6*
CMS024	9	78±3*
CMS034	8	90±5*
CMS036	10	75±9*
IFN-γ	10	80±8*
IL-2	10	80±8*
Saline	10	60±9

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 0.5μg/kg/day, CMS002, CMS011, CMS012, CMS022, CMS028, and CMS035 were found to be able to increase NK cell cytotoxic activity, having statistically significant difference from the saline group (P<0.05). CMS008 was found to be able to inhibit NK cell cytotoxic activity, having statistical significant difference from the saline group (P<0.05). Data detailed in Table 8 below.

Table 8

Group	N	X±SD (%)
CMS002	8	76±9*
CMS008	10	46±12*
CMS011	9	79±3*
CMS012	9	77±6*
CMS022	10	73±11*
CMS028	8	79±3*
CMS035	10	76±10*
IFN-γ	10	72±9*
IL-2	10	74±10*
Saline	11	58±7

* comparing to saline group P<0.05

3. The effect of peptides on the activity of T lymphocyte in secreting IL-2

At 500μg/kg/day, CMS007, CMS009, CMS010, and CMS015 were found to be able to promote IL-2 secretion from T lymphocyte, having statistically significant difference

from the saline group (P<0.05). Among these peptides, CMS007 and CMS015 were found to have statistically significantly difference from the IL-2 group (P<0.05). And, CMS009 and CMS010 were also found to have statistically significant difference from both the IFN-γ group and IL-2 group (P<0.05) as shown in Table 9.

Table 9

Group	N	X±SD (IU)
CMS007	9	86±15*^
CMS009	10	114±13*@^
CMS010	9	125±17*@^
CMS015	9	85±17*^
IFN-γ	10	100±18*
IL-2	10	70±13*
Saline	10	39±10

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 50μg/kg/day, CMS001 and CMS003 were found to be able to promote activity of T lymphocyte in secreting IL-2, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS003 was found to have statistically significant difference from the IL-2 group (P<0.05) as shown in Table 10.

Table 10

Group	N	X±SD (IU)
CMS001	10	60±10*
CMS003	8	86±9*^
IFN-γ	9	99±16*
IL-2	10	72±12*
Saline	10	39±10

* comparing to saline group P<0.05
^ comparing to IL-2 group P<0.05

At 5μg/kg/day, CMS007, CMS012, and CMS020 were found to be able to promote T lymphocyte in secreting IL-2, having statistically significant difference from the saline group (P<0.05) as shown in Table 11.

Table 11

Group	N	X±SD (IU)
CMS007	8	64±12*
CMS012	9	65±16*
CMS020	8	63±11*
IFN-γ	10	96±14*
IL-2	10	77±13*
Saline	10	37±9

* comparing to saline group P<0.05

At 0.5μg/kg/day, CMS010, CMS011, CMS012, CMS022, and CMS034 were found to be able to promote T lymphocyte in secreting IL-2, having statistically significant difference from the saline group ($P<0.05$). Among these peptides, CMS034 was found to have statistically significantly difference from the IL-2 group ($P<0.05$). And, CMS011 and CMS022 were also found to have statistically significant difference from both the IFN-γ group and IL-2 group ($P<0.05$) as shown in Table 12.

Table 12

Group	N	X±SD (IU)
CMS010	9	66±11*
CMS011	10	101±19*@^
CMS012	8	59±13*
CMS022	9	109±14*@^
CMS034	10	85±10*^
IFN-γ	10	87±15*
IL-2	10	73±13*
Saline	10	38±13

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

4. The effect of peptides on the activity of T lymphocyte in secreting IFN

At 500μg/kg/day, CMS010, CMS013, and CMS016 were found to be able to promote T lymphocyte in secreting interferon (IFN), having statistically significant difference from the saline group, IFN-γ group, and IL-2 group ($P<0.05$) as shown in Table 13.

Table 13

Group	N	X±SD (IU)
CMS010	9	167±13*@^
CMS013	9	154±15*@^
CMS016	6	162±19*@^
IFN-γ	10	139±16*
IL-2	10	120±13*
Saline	10	65±11

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 50μg/kg/day, CMS001, CMS003, and CMS021 were found to be able to promote T lymphocyte in secreting IFN having statistically significant difference from the saline

group (P<0.05). Among these peptides, CMS021 was found to have statistically significant difference from the IFN-γ group and IL-2 group (P<0.05) as shown in Table 14.

Table 14

Group	N	X±SD (IU)
CMS001	10	110±15*
CMS003	8	106±16*
CMS021	8	143±17*@^
IFN-γ	9	125±18*
IL-2	10	113±17*
Saline	10	61±11

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 5μg/kg/day, CMS009 and CMS012 were found to be able to promote T lymphocyte in secreting IFN having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS009 was found to have statistically significant difference from the IL-2 group (P<0.05) as shown in Table 15.

Table 15

Group	N	X±SD (IU)
CMS009	10	121±15*^
CMS012	9	86±9*
IL-2	9	105±14*
Saline	10	66±10

* comparing to saline group P<0.05
^ comparing to IL-2 group P<0.05

At 0.5μg/kg/day, CMS010, CMS011, CMS022, and CMS028 were found to be able to promote T lymphocyte in secreting IFN, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS010 and CMS022 were found to have statistically significant difference from the IFN-γ group and IL-2 group (P<0.05) as shown in Table 16.

Table 16

Group	N	X±SD (IU)
CMS010	9	142±18*@^
CMS011	10	89±18*
CMS022	9	145±13*@^
CMS028	10	96±13*
IFN-γ	10	124±16*
IL-2	10	107±13*
Saline	10	64±13

* comparing to saline group P<0.05

@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 groupP<0.05

5. The effect of peptides on antibody formation

At 500μg/kg/day, CMS002, CMS003, CMS007, CMS008, CMS009, CMS010, CMS011, CMS012, CMS013, CMS014, CMS015, CMS016, CMS018, CMS019, CMS020, CMS022, CMS023, CMS024, CMS029, CMS033, and CMS035 were found to be able to promote anti-SRBC antibody formation, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS002, CMS003, CMS007, CMS008, CMS013, CMS019, CMS024, and CMS035 were found to have statistically significant difference from the IFN-γ group (P<0.05). And, CMS009, CMS010, CMS011, CMS012, CMS014, CMS015, CMS016, CMS020, CMS023, CMS029, and CMS033 were also found to have statistically significant difference from both the IFN-γ group and IL-2 group (P<0.05) as shown in Table 17.

Table 17

Group	N	X±SD (Unit)
CMS002	10	87±18*@
CMS003	10	96±18*@
CMS007	10	69±17*@
CMS008	10	82±15*@
CMS009	10	113±22 *@^
CMS010	10	112±30*@^
CMS011	8	188±16*@^
CMS012	8	141±21*@^
CMS013	10	80±16*@
CMS014	10	130±24*@^
CMS015	10	136±22*@^
CMS016	8	143±38*@^
CMS018	10	66±16*
CMS019	10	91±26*@
CMS020	6	155±35*@^
CMS022	8	68±31*
CMS023	9	110±45*@^
CMS024	8	75±29*@
CMS029	8	115±22*@^
CMS033	10	143±27*@^
CMS035	10	88±16*@
IFN-γ	9	37±10
IL-2	10	71±11*
Saline	10	32±7

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 50μg/kg/day, CMS003, CMS011, CMS012, CMS013, CMS015, CMS021, CMS022, CMS023, CMS026, CMS027, CMS029, CMS030, CMS032, CMS033, CMS034, CMS035, and CMS036 were found to be able to promote anti-SRBC antibody formation,

having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS011, CMS013, and CMS015 were found to have statistically significant difference from the IFN-γ group (P<0.05). And, CMS021, CMS022, CMS023, CMS026, CMS027, CMS029, CMS030, CMS032, CMS033, CMS034, CMS035, and CMS036 were also found to have statistically significant difference from both the IFN-γ group and IL-2 group (P<0.05). CMS009 was found to be able to inhibit anti-SRBC antibody formation, having statistical significant difference from the saline group (P<0.05). Data detailed in Table 18 below.

Table 18

Group	N	X±SD (Unit)
CMS003	10	52±11*
CMS009	8	13±5*
CMS011	9	67±9*@
CMS012	8	50±14*
CMS013	8	70±9*@
CMS015	10	54±9*@
CMS021	9	94±20*@^
CMS022	9	110±16*@^
CMS023	8	84±11*@^
CMS026	9	98±9*@^
CMS027	9	93±11*@^
CMS029	10	143±13*@^
CMS030	10	141±33*@^
CMS032	9	131±24*@^
CMS033	8	112±15*@^
CMS034	10	136±11*@^
CMS035	8	97±10*@^
CMS036	10	118±11*@^
IFN-γ	9	37±10
IL-2	10	71±11*
Saline	10	32±7

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 5μg/kg/day, CMS001, CMS003, CMS007, CMS008, CMS009, CMS011, CMS012, CMS013, CMS015, CMS016, CMS019, CMS020, CMS021, CMS023, CMS024, CMS026, CMS027, CMS028, CMS029, CMS030, CMS032, CMS033, CMS034, CMS035, and CMS036 were found to be able to promote anti-SRBC antibody formation, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS003, CMS008, CMS009, CMS012, CMS013, CMS015, CMS016, CMS020, and

CMS021 were found to have statistically significant difference from the IFN-γ group ($P<0.05$). And, CMS001, CMS007, CMS011, CMS019, CMS023, CMS024, CMS026, CMS027, CMS028, CMS029, CMS030, CMS032, CMS033, CMS034, CMS035, and CMS036 were also found to have statistically significant difference from both the IFN-γ group and IL-2 group ($P<0.05$) as shown in Table 19.

Table 19

Group	N	X±SD(Unit)
CMS001	9	110±24*@
CMS003	9	91±24*@
CMS007	9	122±12*@^
CMS008	9	97±26*@
CMS009	8	79±18*@
CMS011	10	115±27*@^
CMS012	10	81±22*@
CMS013	10	93±28*@
CMS015	8	94±37*@
CMS016	9	93±32*@
CMS019	10	118±20*@^
CMS020	10	89±24*@
CMS021	9	82±30*@
CMS023	10	166±27*@^
CMS024	7	171±39*@^
CMS026	9	191±17*@^
CMS027	9	117±45*@^
CMS028	10	121±48*@^
CMS029	9	147±23*@^
CMS030	9	158±37*@^
CMS032	9	157±37*@^
CMS033	7	128±39*@^
CMS034	8	172±37*@^
CMS035	9	176±39*@^
CMS036	8	179±34*@^
IFN-γ	9	37±10
IL-2	10	71±11*
Saline	10	32±7

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 0.5μg/kg/day, CMS021, CMS023, CMS024, CMS027, and CMS033 were found to be able to promote anti-SRBC antibody formation, having statistically significant difference from the saline group ($P<0.05$). Among these peptides, CMS021 and CMS033 were found to have statistically significant difference from the IFN-γ group ($P<0.05$). And, CMS023, CMS024, and CMS027 were also found to have statistically significant difference from both the IFN-γ group and IL-2 group ($P<0.05$). Also, CMS002, CMS003, CMS009, CMS010, CMS011, CMS013, CMS014, CMS015, CMS018, CMS019, CMS020, CMS026, CMS028, CMS029, CMS030, CMS034, and CMS036 were found to be able to inhibit anti-

SRBC antibody formation, having statistically significant difference from the saline group (P<0.05). Data detailed in Table 20 below.

Table 20

Group	N	X±SD (Unit)
CMS002	9	4±1*
CMS003	9	2±1*
CMS009	9	2±1*
CMS010	10	10±3*
CMS011	10	5±3*
CMS013	10	7±1*
CMS014	10	15±6*
CMS015	9	13±4*
CMS018	9	3±1*
CMS019	9	12±3*
CMS020	9	10±3*
CMS021	9	57±9*@
CMS023	10	108±21*@^
CMS024	10	98±6*@^
CMS026	10	19±6*
CMS027	10	99±14*@^
CMS028	10	18±5*
CMS029	9	18±7*
CMS030	9	19±7*
CMS033	9	78±12*@
CMS034	10	20±2*
CMS036	9	20±6*
IFN-γ	9	37±10
IL-2	10	71±11*
Saline	10	32±7

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

6. The effect of peptides on the phagocytotic activity of mononuclear phagocyte

At 500μg/kg/day, CMS003, CMS008, CMS020, CMS022, and CMS024 were found to be able to enhance the phagocytotic activity of mononuclear phagocyte, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS022 was found to have statistically significant difference from the IFN-γ group and IL-2 group (P<0.05) as shown in Table 21.

Table 21

Group	N	X±SD (phagocytotic index)
CMS003	10	6.6±0.7*

CMS008	10	6.5±1.2*
CMS020	10	6.4±0.6*
CMS022	10	7.4±0.6*@^
CMS024	10	6.4±1.0*
IFN-γ	10	6.4±0.9*
IL-2	9	5.7±0.8
Saline	10	5.1±0.6

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 50μg/kg/day, CMS019, CMS024, and CMS030 were found to be able to enhance the phagocytotic activity of mononuclear phagocyte, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS019 was found to have statistically significant difference from the IL-2 group (P<0.05) as shown in Table 22.

Table 22

Group	N	X±SD (phagocytotic index)
CMS019	9	6.7±0.9*^
CMS024	8	6.6±0.7*
CMS030	10	6.3±0.5*
IFN-γ	10	6.4±0.9*
IL-2	9	5.7±0.8
Saline	10	5.1±0.6

* comparing to saline group P<0.05
^ comparing to IL-2 group P<0.05

At 5μg/kg/day, CMS003, CMS008, CMS009, CMS010, CMS011, CMS013, CMS016, CMS018, CMS019, and CMS035 were found to be able to enhance the phagocytotic activity of mononuclear phagocyte, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS003, CMS009, CMS010, CMS016, CMS019, and CMS035 were found to have statistically significant difference from the IL-2 group (P<0.05) as shown in Table 23.

Table 23

Group	N	X±SD (phagocytotic index)
CMS003	9	6.9±0.9*^
CMS008	9	6.4±0.5*
CMS009	9	6.9±0.9*^
CMS010	10	7.1±0.7*^
CMS011	10	6.4±1.1*
CMS013	10	6.7±0.2*
CMS016	9	6.9±0.8*^
CMS018	8	6.7±1.2*

CMS019	8	6.8±0.6*^
CMS035	9	6.9±0.9*^
IFN-γ	10	6.4±0.9*
IL-2	9	5.7±0.8
Saline	10	5.1±0.6

* comparing to saline group P<0.05
^ comparing to IL-2 group P<0.05

At 0.5μg/kg/day, CMS024, CMS027, and CMS036 were found to be able to enhance the phagocytotic activity of mononuclear phagocyte, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS024 was found to have statistically significant difference from the IL-2 group (P<0.05) as shown in Table 24.

Table 24

Group	N	X±SD (phagocytotic index)
CMS024	10	6.7±0.5*^
CMS027	10	6.4±0.6*
CMS036	9	6.2±0.3*
IFN-γ	10	6.4±0.9*
IL-2	9	5.7±0.8
Saline	10	5.1±0.6

* comparing to saline group P<0.05
^ comparing to IL-2 group P<0.05

7. The effect of peptides on the weight of immune organ

At 500μg/kg/day, CMS008, CMS010, CMS016, CMS019, CMS020, CMS022, CMS026, CMS027, CMS028, CMS029, CMS030, CMS032, CMS033, CMS034, CMS035, and CMS036 were found to be able to increase the weight of thymus gland, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS027 and CMS034 were found to have statistically significant difference from the IL-2 group (P<0.05). And, CMS008, CMS022, CMS029, CMS030, CMS032, CMS033, and CMS035 were also found to have statistically significant difference from both the IFN-γ group and IL-2 group (P<0.05) as shown in Table 25.

Table 25

Group	N	X±SD (%)
CMS008	8	0.21±0.03*@^
CMS010	10	0.19±0.04*
CMS016	9	0.18±0.05*
CMS019	10	0.19±0.02*
CMS020	10	0.19±0.04*
CMS022	9	0.26±0.05*@^
CMS026	10	0.20±0.03*^
CMS027	8	0.20±0.03*^
CMS028	10	0.19±0.02*

CMS029	10	0.22±0.04*@^
CMS030	8	0.30±0.03*@^
CMS032	8	0.25±0.03*@^
CMS033	9	0.25±0.04*@^
CMS034	9	0.20±0.05*^
CMS035	10	0.21±0.03*@^
CMS036	9	0.18±0.02*
IFN-γ	10	0.15±0.04
IL-2	9	0.14±0.03
Saline	9	0.12±0.02

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 group P<0.05

At 500μg/kg/day, CMS019 was found to be able to increase the weight of spleen, with statistically significant difference from the saline control groups (P<0.05) as shown in Table 26. CMS001, CMS003, CMS007, CMS009, CMS011, CMS013, CMS014, CMS015, CMS021, CMS023, CMS024, CMS027, and CMS036 were found to be able to decrease the weight of spleen, with statistically significant difference from the saline control group (P<0.05). Data detailed in Table 26 below.

Table 26

Group	N	X±SD (%)
CMS001	10	0.43±0.07*
CMS003	8	0.40±0.04*
CMS007	9	0.32±0.05*
CMS009	9	0.41±0.03*
CMS011	9	0.41±0.04*
CMS013	10	0.44±0.07*
CMS014	10	0.40±0.03*
CMS015	9	0.36±0.07*
CMS019	9	0.63±0.08*
CMS021	9	0.36±0.04*
CMS023	9	0.36±0.06*
CMS024	9	0.34±0.05*
CMS027	10	0.37±0.03*
CMS036	10	0.40±0.03*
Saline	10	0.53±0.05

* comparing to saline group P<0.05

At 50μg/kg/day, CMS002, CMS008, CMS012, CMS014, CMS016, CMS018, CM019, CMS020, CMS022, CMS023, CMS024, CMS026, CMS027, CMS028, CMS029, CMS030, CMS032, CMS033, CMS034, and CMS036 were found to be able to increase the weight of thymus gland, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS034 was found to have statistically significant difference from the IL-2 group (P<0.05). And, CMS002, CMS008, CMS012, CMS014, CMS016, CMS018, CMS019, CMS020, CMS022, CMS023, CMS024, CMS026, CMS027,

CMS030, CMS032, and CMS036 were also found to have statistically significant difference from both the IFN-γ group and IL-2 group ($P<0.05$) as shown in Table 27.

Table 27

Group	N	X±SD (%)
CMS002	10	0.21±0.02*@^
CMS008	10	0.20±0.04*@^
CMS012	10	0.26±0.02*@^
CMS014	10	0.21±0.02*@^
CMS016	10	0.20±0.03*@^
CMS018	10	0.23±0.02*@^
CMS019	10	0.20±0.03*@^
CMS020	10	0.27±0.03*@^
CMS022	10	0.30±0.03*@^
CMS023	10	0.20±0.02*@^
CMS024	10	0.27±0.02*@^
CMS026	10	0.27±0.02*@^
CMS027	8	0.21±0.03*@^
CMS028	10	0.18±0.04*
CMS029	9	0.18±0.05*
CMS030	10	0.25±0.04*@^
CMS032	10	0.27±0.03*@^
CMS033	9	0.18±0.03*
CMS034	8	0.19±0.04*^
CMS036	9	0.22±0.02*@^
IFN-γ	10	0.15±0.04
IL-2	9	0.14±0.04
Saline	9	0.12±0.02

* comparing to saline group $P<0.05$
@ comparing to IFN-γ group $P<0.05$
^ comparing to IL-2 group $P<0.05$

At 50μg/kg/day, CMS008, CMS010, and CMS029 were found to be able to decrease the weight of spleen, with statistically significant difference from the saline control group ($P<0.05$). Data detailed in Table 28 below.

Table 28

Group	N	X±SD (%)
CMS008	10	0.39±0.08*
CMS010	10	0.38±0.05*
CMS029	10	0.42±0.04*
IFN-γ	10	0.50±0.04
IL-2	9	0.62±0.07
Saline	9	0.53±0.05

* comparing to saline group $P<0.05$

At 5μg/kg/day, CMS001, CMS002, CMS010, CMS011, CMS012, CMS013, CMS014, CMS015, CMS016, CMS018, CMS019, CMS020, CMS021, CMS022, CMS023, CMS024, CMS026, CMS028, CMS029, CMS030, CMS032, CMS033, CMS034, CMS 035

and CMS036 were found to be able to increase the weight of thymus gland, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS002, CMS014, CMS024, and CMS030 were found to have statistically significant difference from the IL-2 group (P<0.05). And, CMS010, CMS012, CMS018, CMS019, CMS020, CMS022, CMS026, CMS028, CMS032, CMS034, and CMS036 were also found to have statistically significant difference from both the IFN-γ group and IL-2 group (P<0.05) as shown in Table 29.

Table 29

Group	N	X±SD (%)
CMS001	10	0.22±0.05*
CMS002	9	0.24±0.05*^
CMS010	9	0.27±0.05*@^
CMS011	10	0.22±0.04*
CMS012	10	0.27±0.06*@^
CMS013	10	0.21±0.05*
CMS014	10	0.23±0.06*^
CMS015	9	0.20±0.08*
CMS016	10	0.22±0.06*
CMS018	10	0.24±0.04*@^
CMS019	10	0.24±0.02*@^
CMS020	10	0.24±0.07*@^
CMS021	9	0.20±0.06*
CMS022	9	0.25±0.04*@^
CMS023	10	0.23±0.06*
CMS024	9	0.23±0.06*^
CMS026	10	0.31±0.05*@^
CMS028	10	0.28±0.06*@^
CMS029	10	0.21±0.03*
CMS030	10	0.23±0.07*^
CMS032	10	0.29±0.04*@^
CMS033	10	0.20±0.02*
CMS034	9	0.27±0.06*@^
CMS035	10	0.21±0.04*
CMS036	10	0.25±0.04*@^
IFN-γ	10	0.15±0.04
IL-2	9	0.14±0.04
Saline	9	0.12±0.02

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 groupP<0.05

At 5μg/kg/day, CMS030 was found to be able to increase the weight of spleen, having statistically significant difference from the saline group (P<0.05). CMS015 was found to be able to decrease the weight of spleen, having statistically significant difference from the saline group (P<0.05). Data detailed in Table 30 below.

Table 30

Group	N	X±SD (%)

CMS015	9	0.38±0.15*
CMS030	10	0.64±0.09*
Saline	10	0.53±0.05

* comparing to saline group P<0.05

At 0.5μg/kg/day, CMS002, CMS008, CMS010, CMS012, CMS014, CMS018, CMS020, CMS022, CMS026, CMS028, CMS030, and CMS032 were found to be able to increase the weight of the thymus gland, having statistically significant difference from the saline group (P<0.05). Among these peptides, CMS008 and CMS012 were found to have statistically significant difference from the IL-2 group (P<0.05). And, CMS002, CMS020, and CMS030 were also found to have statistically significant difference from both the IFN-γ group and IL-2 group (P<0.05) as shown in Table 31.

Table 31

Group	N	X±SD (%)
CMS002	8	0.26±0.06*@^
CMS008	10	0.22±0.07*^
CMS010	9	0.21±0.03*
CMS012	10	0.22±0.06*^
CMS014	10	0.20±0.04*
CMS018	10	0.20±0.03*
CMS020	9	0.23±0.05*@^
CMS022	10	0.21±0.06*
CMS026	9	0.21±0.05*
CMS028	10	0.20±0.06*
CMS030	8	0.24±0.05*@^
CMS032	10	0.21±0.06*
IFN-γ	10	0.15±0.04
IL-2	9	0.14±0.04
Saline	9	0.12±0.02

* comparing to saline group P<0.05
@ comparing to IFN-γ group P<0.05
^ comparing to IL-2 groupP<0.05

At 0.5μg/kg/day, CMS020 was found to be able to increase the weight of spleen, having statistically significant difference from the saline group, IFN-γ group, and IL-2 group (P<0.05). CMS001 was found to be able to decrease the weight of spleen, having statistically significant difference from the saline group (P<0.05). Data detailed in Table 32 below.

Table 32

Group	N	X±SD (%)
CMS001	10	0.40±0.05*
CMS020	8	0.68±0.09*@^
Saline	10	0.53±0.05
IFN-γ	10	0.50±0.04
IL-2	10	0.62±0.07

* comparing to saline group $P<0.05$
@ comparing to IFN-γ group $P<0.05$
^ comparing to IL-2 group $P<0.05$

In summary, we found that peptides CMS001, CMS002, CMS003, CMS007, CMS008, CMS009, CMS010, CMS011, CMS012, CMS013, CMS014, CMS015, CMS016, CMS018, CMS019, CMS020, CMS021, CMS022, CMS023, CMS024, CMS026, CMS027, CMS028, CMS029, CMS030, CMS032, CMS033, CMS034, CMS035, and CMS036 have in vivo biological activities in the animal model tested.

II. <u>THE IN VIVO ANTIVIRAL EFFECT OF PEPTIDES</u>

In order to find out whether these peptides have possible therapeutic effect on viral infections, we used the animal model duck hepatitis B infection in this study to test out the in vivo effects of the above peptides on diseased animals.

Chongqing duck hepatitis B model was set up and treated with peptides by intraperitoneal injection (50μg/kg/day, once per day) for 4 weeks. The serum level of DHBV DNA was analyzed by serum dot-blot hybridization. Lamivudine and normal saline treatment was used as positive and negative control respectively. The peptide CMS001 was found to be able to reduce the serum level of DHBV DNA at the 4th week of the treatment, having statistically significant difference from the saline control group ($p<0.05$). It is concluded that CMS001 at suitable dosage level can be used as a part, or on its own, for viral hepatitis infection management.

Materials and methods

1. Peptides were custom synthesized by American Peptide Company, Inc., USA, from L-amino acids.

2. Animal model [1]

One-day old Chongqing ducks were inoculated with 0.1ml stock serum positive of Duck Hepatitis B Virus (DHBV) DNA (5×10^7 copy/ml) by intraperitoneal injection. One week later, blood samples were collected from the external jugular vein and infection was confirmed by dot-blot hybridization with DHBV DNA probe labeled with digoxin [2]. The ducks were breed to 2 weeks old for entrance into the study.

3. Grouping and treatment

DHBV infected ducks were randomized into the following groups:

a) Negative control group (n=9):	Normal saline was given 1ml per duck by intraperitoneal injection, once per day.
b) Lamivudine group (n=8):	As positive control group. Lamivudine [4] was given 50mg/kg/day by oral administration, once

per day.

c) Peptide group (n=9): 50μg/kg/day peptide (adjusted to final volume of 0.5 to 1ml with normal saline) was given once per day by intraperitoneal injection.

Treatment lasted for 4 weeks and observation continued for another one week after termination of treatment. 1 ml blood samples were drawn from the external jugular vein of the ducks on days 0, 7, 14, 21, 28, and 35 when treatment started. Sera of the blood samples were isolated immediately [3] and stored at -20°C until analysis.

4. Serum DHBV DNA level determinations

DHBV DNA probe was fluorescent labeled according to the labeling kit protocol from the kit manufacturer (Amersham Pharmacia Biotech Co.). 40μl duck sera were dot-blotted (1 duplicated dot per sample) onto nitro-cellulose membrane and hybridized with fluorescent-labeled DHBV DNA probe for quantitation [2]. After completion of hybridization, the blots were developed in CDP-Star fluorimetry reagent RPN3690 and scanned with Vuego Scan (Brisa-620st) scanner. ImageMaster TotalLab v1.11.Ink software was used for quantitative analysis of the blots. Statistical analysis was carried out according to the pair t-test with SPSS software.

Results

Table II.1 Serum DHBV DNA titer before and after treatment

	DHBV DNA level (Mean ± Standard Deviation, 10^3 units)					
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
Normal saline	26±12	45±31	49±23	102±66	60±38	50±43
Lamivudine	21±9	6±4*	7±6*	8±7*	8±5*	20±19
CMS001	21±18	11±13	20±18	14±14	5±3*	18±16

Pair t-test, comparing with day 0 of the same animals: *P<0.05

The negative (normal saline) and positive (lamivudine) control group proved successful establishment of the hepatitis animal model. At 50μg/kg/day, the peptide CMS001 was found to be able to decrease the serum DHBV DNA titre after 4 weeks of treatment, having statistically significant difference (p<0.05) from the before treatment value of the same animals. Upon termination of treatment, the serum DHBV DNA titre rebounded to a value with no statistical difference from that before treatment, showing that the effect of peptide CMS001 may be reversible and/or need longer treatment period for eradication of the virus.

Discussion

Duck hepatitis animal model [1] is an established experimental model for human hepatitis B pathogenesis studies and for the screening of hepatitis B therapeutic agents. In this study, CMS001 was found to be able to decrease the serum titre of DHBV DNA after 4 weeks of treatment, indicating that the peptide CMS001 at suitable dosage level and with suitable application scheme, can be useful on its own or in combination with other substances as an agent for human hepatitis B management.

In the present study, administration by intraperitoneal injection was tested, but this does not exclude the possible effectiveness of the peptide if administered via other alternative routes. The peptides may also be administered via intravenous injection, intramuscular injection, subcutaneous injection, and subcutaneous implantation, with or without delivery facilitating device such as liposome, sustain release protection etc. The peptide may also be administered in any form of oral administration like tablet, capsule, suspension, solution etc, in the usual form without modification or in slow release form, or with or without gastro-enteric protection. The peptide may further be applied in any form of topic application like ointment, cream, gel etc with or without transdermal facilitating device, or as inhalant of powder, dissolved, or as liposome protected form. The peptide may also be interpreted into its genetic sequence and cloned into an expression system, on its own or in combination with other peptide sequences, to generate a resulting peptide molecule to make use of the activity of the peptide as described in this report, with or without purification of the resulting peptide.

Table II.2 Peptides Effective for Hepatitis B

CMS code	SEQ ID No.
CMS001	1

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III. EFFECT OF PEPTIDES ON NEPHRITIS

In order to find out whether these peptides have possible therapeutic effect on nephritis, we used the animal model rat Masugi nephritis in this study to test out the in vivo effects of the above peptides on diseased animals [3,4].

The objective of this study is to investigate the in vivo therapeutic effect of peptides on chronic glomerulonephritis. Masugi nephritis rat model was constructed by injecting rabbit anti-rat-renal-cortex IgG into healthy Sprague Dawley rats, and then treated immediately with intraperitoneal injection of peptides at 50μg/kg/day, once per day for 3 weeks. Hydrocortisone was used as positive control. It was found that proteinurea, serum creatinine level, and splenic index of the rats treated with CMS014, CMS018, CMS030, and CMS036 were lowered compared with that of the control group, with statistical significance ($p<0.05$). Postmortem microscopic examination of the kidney showed that the therapeutic effect of these peptides was similar to hydrocortisone treatment. It is concluded that CMS014, CMS018, CMS030, and CMS036 may be used as a means for chronic nephritis management.

Materials

Sprague Dawley (SD) rats, male, weighing 120±20g, were from Center of Experimental Animal, Guangzhou University of Traditional Chinese Medicine, and also from First Military Medical University. Chinchilla rabbits weighing 3kg were from Center of Experimental Animal, Guangzhou University of Traditional Chinese Medicine.

Peptides, of L-amino acids origin, were custom synthesized by American Peptide Company, Inc, USA, and were diluted to 10μg/ml in sterile normal saline. Hydrocortisone was from Yangzhou Pharmaceutical Factory, China.

Serum creatinine level determination kit from Shanghai Rongsheng Biological Technique Company, PR China.

Methods

1. Preparation of rabbit anti-rat-renal-cortex anti-serum [1]: 20 healthy SD rats were anesthetized with intravenous injection of 3% sodium pentobarbital, 40mg/kg. The aorta abdominalis was exposed and the kidneys were perfused with normal saline until clean of blood. The renal cortex was isolated, homogenized in 5 volumes of 0.01M Tris-HCl buffer, pH 8.1, and filtered through a 140-gauge stainless steel mesh. The filtrate was collected and mixed with either GIBCO-RRE complete or incomplete Freunds adjuvant in 1:1 and emulsified to obtain the working immunization solution.

10 healthy rabbits were immunized with the immunization solutions, first time with complete Freunds adjuvant and subsequently with the incomplete one. The antigen was

injected hypodermally through 6 random points, 0.1 ml per point, once per 10 days. From 6^{th} week onwards, blood was collected from the auricular vein and the antibody titer was determined by the double diffusion method [2]. On the 8^{th} week after starting of immunization, the rabbits were anesthetized with intravenous injection of 3% sodium pentobarbital, 20mg/kg, and blood was collected from the carotid artery. The anti-serum was then exuded and isolated.

Whole blood was collected from 15 healthy SD rats. The red blood cell was isolated and washed clean thrice with normal saline. The clean red blood cell was mixed with 250 ml rabbit anti-serum and incubated at 4°C overnight. After incubation, the blood cell was removed by centrifugation and the supernatant was further inactivated at 56°C for 30 minutes. After centrifugation to remove any precipitate, crude IgG was isolated and partially purified from the supernatant by precipitation with $(NH_4)_2SO_4$ thrice (50%, 33%, and then 33%) [5]. The crude IgG was redissolved in 125ml double distilled water and dialysed clean of ammonium sulphate. The anti-rat-renal-cortex antibody titer was determined by the double diffusion method [2].

2. Induction of rat glomerulonephritis [5]: 0.25ml priming solution (containing about 4mg non-specific rabbit IgG with complete Freunds adjuvant) was injected intraperitoneally into healthy SD rats to prime the rats five days before induction. With the exception of the normal healthy control group that received normal saline, all groups were subsequently induced by intraperitoneal injection of 1ml rabbit anti-rat-renal-cortex IgG prepared as described in section Method 1.

3. Grouping and administration: male SD rats were randomized into groups of normal healthy control (normal), nephritis placebo treatment control (placebo), nephritis peptide treatment, and the nephritis hydrocortisone treatment control (hydrocortisone), 12 per group. Treatment started on the day of induction. Peptides 50μg/kg/day, hydrocortisone 3.3mg/kg/day, and normal saline was used as placebo, all administered intraperitoneally once per day and lasted for 3 weeks.

4. Parameters monitored [4]:

(a) urine protein level: each rat was kept individually in one cage. Adequate drinking water was supplied. Urine was collected once per week, 24 hours per time. Protein content of the urine was determined by the Coomassie blue method.

(b) serum creatinine level: blood was collected after three weeks of treatment and serum creatinine level determination. The creatinine kit was provided by Shanghai Rongsheng Biological Technique Company.

(c) Spleen weight index: spleen weight index was determined by the following formula:

Spleen weight index = mean spleen weight ÷ mean body weight

(d) Pathological microscopic examination of kidney: 6 heaviest kidneys were chosen from each group for pathological examination.

Statistical analysis

Used t-test for inter-group comparisons, cut off set at $p<0.05$. For all groups, the two rats with lowest urinary protein levels were excluded from the statistical analysis.

Result

1. The effect of peptide treatment on the urine protein level

Table III.1 The effect of peptide treatment on urine protein level (unit:mg)

Groups	n	Week 1	Week 2	Week 3
CMS014	10	5.5±4.0*	6.3±6.8*	2.8±1.9*
CMS018	10	7.0±3.7*	5.5±7.9*	3.3±3.4*
CMS030	10	5.4±3.4*	9.5±16.2	2.4±1.5*
CMS036	10	7.9±5.8*	5.0±7.1*	1.3±0.9*
hydrocortisone	10	3.1±2.0*	7.5±7.7	7.6±7.1*
placebo	10	22.9±22	17.2±14.5	20.2±29.0
normal	9	1.7±1.3*	2.3±1.1*	0.4±0.2*

Comparing with placebo, * $p<0.05$

It was found that peptides CMS014, CMS018, CMS030, and CMS036 at 50μg/kg/day once per day can lower the urine protein level of Masugi nephritis rat model, with statistically significant difference from the placebo treated control group, $p<0.05$. It was also noted that the growth rate of groups CMS014, CMS030, CMS036, and hydrocortisone have slower growth rate than normal. The growth rate of the hydrocortisone group decreased to such an extent that after the first week, the treatment dose has to be halved to avoid severe intolerance.

2. The effect of peptide treatment on the serum creatinine level

Table III.2 The influence of peptides on the serum creatinine level

Groups	n	Serum creatinine level (μmol/L)
CMS014	10	159±1
CMS018	10	67±1*
CMS030	10	93±1*
CMS036	10	80±1*
Placebo	10	265±212
hydrocortisone	10	239±107
normal	9	80±1*

Comparing with nephritis rats receiving placebo treatment (placebo), * $p<0.05$

The serum creatinine level of Masugi nephritis rats receiving placebo treatment was much higher than that of the normal control group, showing that the renal function of the nephritis rats was abnormal. It was found that the peptides CMS014, CMS018, CMS030, and CMS036 at 50µg/kg/day once per day were able to lower the level of serum creatinine, with statistically significant difference from the nephritis rats with placebo treatment.

3. The effect of peptides on the spleen indexes

Table III.3 The effect of peptides on the spleen index($x10^{-3}$)

Groups	n	spleen index
CMS014	10	3.6±1.3*
CMS018	10	3.3±0.8*
CMS030	10	3.0±0.5*
CMS036	10	3.4±0.8*
placebo	10	4.8±1.1
hydrocortisone	10	4.5±1.4
normal	9	2.4±0.1*

Comparing with nephritis rats receiving placebo treatment (placebo), * $p<0.05$

The spleens of all induced rats were enlarged compared with normal rats, showing that the induction of nephritis was related to immune response. It was found that the peptides CMS014, CMS018, CMS030, and CMS036 at 50µg/kg/day once per day were able to decrease the spleen index, with statistically significant difference from the group of nephritis rats receiving placebo treatment, $p< 0.05$. This suggested that the peptides might exert their corrective effect against nephritis through immunosuppression mechanisms.

4. The effects of peptides on the renal pathological microscopic examination

Comparing with normal rats, the nephritis rats receiving placebo treatment (placebo group) showed signs of fibrous tissue formation in the glomerular capsule, hyperplasia of the glomerular epithelium, formation of crescents, dilation and congestion of glomerular capillaries, edema of the proximal tubule epithelium, and cast formation in the distal tubule and collecting duct. These pathological changes confirmed the successful induction of nephritis. It was observed that in the group CMS014, there was only one rat showed signs of fibrous tissue formation in the glomerular capsule and hyperplasia of the glomerular epithelium. Other parameters of the same rat, and other rats of the same group have essentially the same renal histology as normal rats. In the groups CMS018, CMS030, and CMS036, all pathological parameters of all rats were normal.

Conclusion

It is concluded that the peptides CMS014, CMS018, CMS030, and CMS036 at 50μg/kg/day, once per day, administered intraperitoneally can have therapeutic effect on experimental Masugi nephritis rat model. Proteinurea, creatinine excretion, and renal histology of the treated rats were corrected by these peptides, with statistically significant difference from the control group receiving placebo treatment. These peptides may act through immunological mechanisms as indicated by their influence on the spleen weight index, but does not exclude the possibility of their actions via other mechanisms.

Discussion

The peptides CMS014, CMS018, CMS030, and CMS036 may be useful as a part, or on its own, in nephritis management in human. For example, the peptides may be used to correct proteinurea or to restore the excretory functions of nephritis patients. The peptides may also be used to prevent further kidney function impairment in nephritis patients. The peptides may be used on its own, in combinations of two or more peptides, or in combination with other pharmaceuticals or food supplements as a total course for nephritis management.

Table III.4 Peptides Effective for Nephritis

CMS code	SEQ ID No.
CMS014	7
CMS018	10
CMS030	21
CMS036	26

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IV. EFFECT OF CMS PEPTIDES ON CANCER

In order to find out whether these peptides have possible therapeutic on cancer, we used various standard animal cancer models in this study to test out the biological effects of the above peptides on diseased animals.

Materials

1. Experimental animals

BALB/c mice, $C_{57}BL/6$ mice, and DBA/2 mice, weighing 18-22g, from China Medical Science Institute, PR China.

2. Cell lines

Mouse sarcoma S_{180} cells, B_{16} cells, and L_{1210} cells, from Cancer Research Department, China Medical Science Institute.

YAC-1 cells, as gift from Prof. Yao Zhi, Tianjin Medical University.

3. Main drug and reagent

The peptides used in this study were custom manufactured by American Peptide Company, Inc., USA.

Fetal bovine serum, RPMI-1640 cell culture medium, from Gibco, USA.

MTT, ConA, from Sigma, USA.

Recombinant mouse interferon-γ (rmIFN-γ), from Beijing Biotech Inc., PR China.

Recombinant human interleukin-2 (rhIL-2), from Shanghai Huaxin Biotech Inc., PR China.

Lymphocyte separation solution, from Research Institute of Hematologic Disease, National Institute of Medical Science, PR China

Cyclophosphamide, from The 12th pharmaceutical factory of Shanghai, PR China.

METHODS

1. Administration of test substance

Intraperitoneal injection, once per day. With the exception of cyclophosphamide group, all groups started treatment 5 days before transplanting the cancer cells. The cyclophosphamide groups were treated on the next day after transplanting the cancer cells. Treatment of all groups with testing substance lasted for 30 days or until the animal died unless otherwise specified.

2. The effect of peptides on the growth rate of transplanted S_{180} sarcoma cells in BALB/c mice, and that on the immunological function of the host

BALB/c mice were randomized into peptide group, cyclophosphamide group, rmIFN-γ group, rhIL-2 group, and saline group, 20 animals per group.

Stock S_{180} sarcoma cells were incubated in DMEM/F12 medium supplemented with 10% fetal bovine serum, 37°C, 5%CO_2 for 72 hrs, then washed 3-4 times with Hank's solution at room temperature. Adjust the cell concentration to 1-2 x 10^9 per litre with Hank's solution. 0.2ml of cell suspension was implanted to several BALB/c mice through the armpit for 10-12 days. The mice were killed by cervical vertebra dislocation. Vigorously growing and non-disrupted tumor masses were harvested and washed clean with sterile saline. The tissue was dispersed in saline to a homogenous cell suspension, in a ratio of 1g tissue to 4 ml saline. The sarcoma bearing mice model was prepared with an injection of 0.2ml cell suspension through the armpit [1]. Administration of test substance treatment started as described in section Method 1.

2.1 The effect of peptides on the phagocytotic function of mononuclear phagocyte in mice with S_{180} sarcoma [2,3] was analysed by injecting mice from the tail vein with 0.1ml/10g body weight of Indian ink (1:5 dilution with normal saline) on the second day after the last test substance administration. At one minute and five minutes after the injection, 20μl blood was obtained from the eye canthus with heparinised tubing. The blood was mixed with 2ml 0.1% w/v Na_2CO_3 and then OD_{680nm} obtained. The outline clear index K was calculated by the following formula:

$$K = (\lg A_1 - \lg A_2) \div (t2 - t1)$$

Key:

A1: OD_{680}nm at first minute

A2: OD_{680}nm at fifth minute

t2: 5 minutes

t2: 1 minute

After the phagocytosis index study, the mice were killed by cervical vertebra dislocation. The liver, spleen, and cancer tissue were dissected, blotted dry, and weighed.

The phagocytosis index α was calculated as below:

$$\alpha = (\sqrt[3]{K}) \times (W \div W_{LS})$$

key:

W: body weight

W_{LS}: weight of liver plus spleen

2.2 The tumor growth inhibition index was calculated according to the following formula:

Tumor growth inhibition index = (mean tumor weight of control group – mean tumor weight of treatment group) ÷ mean tumor weight of control group

3. <u>The effect of peptides on the survival of BALB/c mice with transplanted ascitic fluid type liver cancer H_{22}</u>

BALB/c mice were randomly grouped into peptide group, cyclophosphamide group, rmIFN-γ group, rhIL-2 group and saline group, 20 animals per group.

Stock H_{22} cells were incubated in DMEM/F12 medium supplemented with 10% fetal bovine serum, 37°C/5%CO_2 for 72 hrs, then washed 3-4 times with Hank's solution at room temperature. Adjust cell concentration to 1-2 x 10^9 per litre with Hank's solution. 0.2ml of the cell suspension was implanted to the abdominal cavity of several BALB/c mice for 6-8 days [1]. The mice were killed by cervical vertebra dislocation. Ascitic fluid of the mice was collected aseptically and the cell concentration adjusted to 1 x 10^6 per ml with Hank's solution. 0.2ml of the cell suspension was implanted into the abdominal cavity of healthy mice to generate the H_{22} carrying mouse model for ascitic fluid type liver cancer. Administration of test substance started as described in section Method 1. Survival data of the mice was recorded. If the animal survived longer than the experiment, the survival days was recorded as the duration of the experiment. Mean survival day was obtained by the Kaplan-meier method in the Survival option of the SPSS software. The survival index was calculated according to the following formula:

Survival index = (mean survival days in treatment group – mean survival days of control group) ÷ mean survival days of control group x 100%

4. <u>The effect of peptides on the cellular immunity of BALB/c mice with transplanted ascitic fluid type liver cancer H_{22}</u>

4.1 Preparation of spleen cell suspension [1,4]

Healthy BALB/c mice were randomised into peptide group, rmIFN-γ group, rhIL-2 group, and saline group, 15 mice per group, and prepared into H_{22} carrying mice model as described in section Method 3. After implantation of the cancer cells, the test substances were administered for 15 days, the mice were killed by cervical dislocation. The spleen was isolated aseptically and manually dispersed in cold D-Hank's solution using an injection needle. The dispersed cell suspension was further sieved through a 100-gauge 150μm diameter stainless steel sieve. After centrifugation at 200g for 10 minutes, the supernatant was discarded. The cell pellet was re-suspended in 10 volume of Tris-NH_4Cl buffer and then kept standing still for 10 minutes at room temperature. The suspended cells were collected by centrifugation at 150g for 10 minutes. The cells were washed 2-4 times with cold D-Hank's solution by re-suspending and collecting by centrifugation with condition as described above. The washed cells were then diluted to the desired cell densities by RPMI-1640 culture medium, containing 10% fetal bovine serum.

4.2 The effect of peptides on the T lymphocyte transformation in mice with ascitic fluid type liver cancer H_{22} [1,4]

Spleen cells of density 1×10^6/ml were placed onto a 96 wells cell culture plate, 100µl/well, three parallel wells each of the assay sample and control sample per mouse. To the assay wells, 100µl/well ConA of 100µg/ml in RPMI-1640 was added, and 100µl/well plain RPMI-1640 was used for the controls. The cells were incubated for 66 hrs at 37°C, 5% CO_2. The cells were then pelleted by centrifugation at 150g for 10 minutes. The supernatant was collected and stored at –20°C for cytokines IL-2 and IFN determination.

50µl/well MTT of 1mg/ml in RPMI-1640 was added to the cell pellet and the cells re-suspended by shaking for 2 minutes. The incubation was continued for 4 hours. The supernatant was discarded after centrifugation at 150g for 10 minutes. 120µl 40mM HCl-2-propanol was added to the cell pellet and shaken for 3 minutes. Use an ELISA reader to obtain OD_{570}nm of each well referenced at 630nm.

Each mouse formed three assay wells and three control wells. The Stimulation Index (SI) of each mouse was obtained by first deriving the average OD of the three parallel wells, then dividing the value of the assay wells by the control wells.

4.3 The effect of peptides on the NK cell activity in mice with ascitic fluid type liver cancer H_{22} [5, 6]

Mice spleen cells were prepared to $4\text{x}10^6$/ml as described in section 4.1 above. Target cells YAC-1 were brought to log phase and adjusted to $1\text{x}10^5$/ml. Using a 96 wells cell culture plate, 100µl mouse spleen cells and 100µl culture medium were added to the control well containing only the spleen cells; 100µl target cells and 100µl culture medium were added to the control well containing only target cells; 100µl mouse spleen cells and 100µl target cells were added to the NK activity assay well. Three parallel sets of the above were prepared per mouse. After that, the 96 wells cell culture plates were incubated for 4hrs at 37°C, 5% CO_2.

Samples were centrifuged at 150g for 10 minutes to collect the cells. The supernatant was discarded and 50µl/well MTT of 1mg/ml was added. The reaction_mixture was then shaken for 2 minutes, and incubated at 37°C, 5% CO_2 for 4 hours. The supernatant was discarded after centrifugation at 150g for 10 minutes. 120 µl 40mM HCl-2-propanol was added and shaken for 3 minutes. An ELISA reader was used to obtain OD_{570nm} of each well referenced at 630nm.

Each mouse has 9 wells: three spleen cells only control, three target cells only control, and three assay wells with both spleen and target cells. The NK cell activity index of each mouse was obtained by first deriving the average OD of the three parallel wells of each combination, then applying this average OD to the following formula:

NK cell activity index = [1-(average OD of spleen and target cell well – average OD of spleen cell only well) ÷ (average OD of target cell only well)] x 100%

5. The effect of peptides on the survival of DBA/2 mice with transplanted L_{1210} leukemia

DBA/2 mice, 6-8 weeks old, were randomized into peptide group, cyclophosphamide group, rmIFN-γ group, rhIL-2 group, and saline group, 20 animals per group.

Stock L_{1210} cells were incubated in DMEM/F12 medium supplemented with 10% fetal bovine serum, 37°C/5%CO_2 for 72 hrs, then washed 3-4 times with Hank's solution, and adjusted to 1 x 10^5 cells per litre. 0.1ml of the cell suspension was implanted into the abdominal cavity of several healthy DBA mice for 6-8 days. The mice were then killed by cervical vertebra dislocation and their ascitic fluid collected aseptically. The cell concentration of the collected ascitic fluid was adjusted to 1 x 10^5 per ml with Hank's solution. 0.1ml of the cell suspension was implanted into each of the testing animal and the survival data of the animals recorded. Treatment started in the testing animals as described in section Method 1. Mean survival day was obtained by the Kaplan-meier method in the Survival option of the SPSS software. If the animal survived longer than the experiment, the survival days was entered as the duration of the experiment. The survival index was calculated according to the following formula:

Survival index = (mean survival days of treatment group – mean survival days of control group) ÷ mean survival days of control group

6. The effect of peptides on the humoral immunity of C_{57}BL/6 mice bearing transplanted B_{16} melanoma, and that on the metastatic potential of the inoculated melanoma cells

C_{57}BL/6 mice, 6-8 weeks old, body weight 18-22g, were randomized into peptide group, cyclophosphamide group, rmIFN-γ group, rhIL-2 group, and saline group, 20 animals per group.

Stock B_{16} mouse melanoma cells were incubated in DMEM/F12 medium supplemented with 10% fetal bovine serum, 37°C/5%CO_2 for 72 hrs, then washed 3-4 times with Hank's solution. The cell concentration was adjusted to 1 x 10^5 per ml with Hank's solution and 0.1ml of the cell suspension was injected via the tail vein into the testing mice to generate the B_{16} melanoma bearing animal model [7,8]. Treatment with test substance started as described in section Method 1.

6.1 The effect of peptides on the humoral immunity of C_{57}BL/6 mice bearing transplanted B_{16} melanoma [9]

Sheep red blood cells (SRBC) were prepared by collecting blood from the cervical vein and put into a sterile flask with glass beads. The flask was shaken for 3 minutes and

the blood then mixed with Alsever solution (glucose 2.05g, NaCl 0.4g, Na lemonade 0.8g, adjust to 100ml with distilled water) and stored at 4°C. Immediately before use, samples were centrifuged at 130g, 5 minutes to collect the SRBC. The cells were washed two times by re-suspension and centrifugation in normal saline. Then the cell pellet was collected by centrifugation at 180g for 10 minutes and re-suspended in saline to make the final working SRBC suspension, 2% (v/v).

Complement was prepared by adding 10 volumes of fresh Cavy serum into one volume centrifuge packed SRBC, and then gently shaken for 30 minutes at 4°C. The SRBC was removed by centrifugation at 200g for 10 minutes. 10 volumes of normal saline were added to obtain the working complement solution.

On the testing animals, on the 27th date of test substance treatment, 0.2ml of the working SRBC cell suspension was injected into each animal to raise antibody. On the day after the last test substance administration, blood was collected from the eye canthus and left at room temperature for one hour for serum exudation. After centrifugation at 200g for 10 minutes, the collected serum was diluted by 500 times with normal saline.

To 1ml diluted mouse serum of each mouse, 0.5ml SRBC suspension was added. Ice cold. Then 1ml working complement solution was added and incubated at 37°C water bath for 10 minutes. Reactions were terminated by ice cold. Samples were then centrifuged at 200g for 10 minutes to obtain the supernatant.

To 1ml of this supernatant, 3ml Drabkin solution was added and left at room temperature for 10 minutes. OD_{540nm} was obtained.

Reference OD_{540nm} was obtained by mixing 0.25ml SRBC suspension with Drabkin solution to 4ml and placed at room temperature for 10 minutes before OD_{540nm} was taken.

Hemolysis index = (OD_{540nm} of test sample ÷ reference OD_{540nm}) x 500

6.2 After the humoral immunity study, the mice were killed by cervical vertebra dislocation. Postmortem examination of the animals was performed. The pathological changes were recorded, and the numbers of melanoma lung metastasis foci were counted.

Results

1. <u>The effect of peptide on the cellular phagocytosis in BALB/c mice with transplanted S_{180} sarcoma (Method 2.1)</u>

Table IV.1. The effect of peptide on the phagocytosis index of BALB/c mice with transplanted S_{180} sarcoma

Group	Dose	N	Phagocytosis index

CMS001	50μg/kg	20	6.24±0.33*^
CMS001	5μg/kg	19	6.67±0.43*^
CMS034	5μg/kg	19	6.20±0.44*^
CMS034	0.5μg/kg	20	6.35±1.02*
IL-2	3×10^5IU/kg	19	6.96±1.37*
IFN-γ	3×10^5IU/kg	17	5.45±0.71
Cyclo-phos phamide	20mg/kg	19	5.92±2.47
Saline	0.5ml	19	5.38±0.85

*: comparing to saline, P<0.05
^: comparing to rmIFN-γ, P<0.05

CMS001 at 50μg/kg/day and 5μg/kg/day, and CMS034 at 5μg/kg/day and 0.5μg/kg/day were found to be able to increase the phagocytosis index, having statistically significant difference from the saline group.

2. The effect of peptide on the growth rate of transplanted S_{180} sarcoma in BALB/c mice (Method 2.2)

Table IV.2. The effect of peptide on transplanted S180 tumor growth

Group	Dose	N	Tumor weight(g)	Tumor inhibition index (%)
CMS010	500μg/kg	20	0.67±0.35*	48.4
CMS034	0.5μg/kg	20	0.83±0.48*	35.9
CMS035	5μg/kg	20	0.71±0.37*	44.6
IL-2	3×10^5IU/kg	20	0.69±0.37*	46.2
IFN-γ	3×10^5IU/kg	18	0.96±0.45	25.3
cyclo-phos phamide	20mg/kg	20	0.68±0.32*	47.3
Saline	0.5ml	20	1.29±0.50	

* comparing to saline group, P<0.05

CMS010 at 500μg/kg/day, CMS034 at 0.5μg/day, and CMS035 at 5μg/kg/day were found to be able to reduce the growth of the transplanted S_{180} sarcoma, having statistically significant difference from the saline group ($P < 0.05$).

3. The effect of peptide on the survival of BALB/c mice with transplanted ascitic fluid type liver cancer H_{22} (Method 3)

Table IV.3. The effect of peptide on the survival index of BALB/c mice with transplanted ascitic fluid type liver cancer H22

Group	Dose	N	Survival days	Survival index (%)
CMS008	5μg/kg	20	50.7±20.9*&	67.8
CMS011	5μg/kg	20	36.4±22.2*&	60.2
CMS024	50μg/kg	20	36.3±12.7*&$	38.4
CMS024	5μg/kg	19	40.6±14.6*&$	54.8
CMS024	0.5μg/kg	19	46.4±14.8*^&$	76.9
CMS032	0.5μg/kg	20	42.8±12.2*^&$	63.3
rhIL-2	3×10^5IU/kg	18	13.6±0.5	
rmIFN-γ	3×10^5IU/kg	20	27.8±7.5	6.1
Cyclo-phos phamide	20mg/kg	20	24.7±10.2	
Saline	0.5ml	19	26.2±6.8	

*: comparing to saline, P<0.05
^: comparing to rmIFN-γ, P<0.05
&: comparing to rhIL-2, P<0.05
$: comparing to cyclophosphamide, P<0.05

CMS008 at 5μg/kg/day, CMS011 at 5μg/kg/day, CMS024 at 50μg/kg/day, CMS024 at 0.5μg/kg/day, and CMS032 at 0.5μg/kg/day were found to be able to prolong the survival of BALB/c mice with transplanted H_{22} ascitic fluid type liver cancer, having statistically significant difference from the saline group ($P<0.05$). It was also observed that in the group CMS024 0.5μg/kg/day, more than 30% (n=6) of the mice could survive longer than 90 days (two months after the experiment ended). Postmortem examination of the mice did not show sign of tumor establishment. CMS024 at 0.5μg/kg/day therefore may interfere with the

growth of the transplanted H_{22} by interfering with its establishment or inducing the complete healing of the established cancer.

4. The effect of peptide on T lymphocyte transformation in BALB/c mice with transplanted ascitic fluid type liver cancer H_{22} (Method 4.2)

Table IV.4. The effect of peptide on T lymphocyte transformation

Group	Dose	N	Stimulation index
CMS010	500μg/kg	20	1.45±0.21*$
CMS019	0.5μg/kg	19	1.50±0.19*$
CMS024	0.5μg/kg	19	1.46±0.19*$
CMS024	5μg/kg	20	1.45±0.21*$
CMS034	0.5μg/kg	20	1.37±0.10*$
CMS035	0.5μg/kg	20	1.40±0.13*$
CMS035	5μg/kg	20	1.46±0.16*$
rhIL-2	3×10⁵IU/kg	19	1.46±0.21*
rmIFN-γ	3×10⁵IU/kg	18	1.27±0.14
Cyclo-phos phamide	20mg/kg	19	1.01±0.23*
Saline	0.5ml	20	1.25±0.07

*: comparing to saline, P<0.05
$: comparing to cyclophosphamide, P<0.05

CMS010 at 500μg/kg/day, CMS019 at 0.5μg/kg/day, CMS024 at 0.5μg/kg/day and 5μg/kg/day, CMS034 at 0.5μg/kg/day and CMS035 at 0.5μg/kg/day and 5μg/kg/day were found to be able to increase the stimulation index of T-lymphocyte, having statistically significant difference from the saline group (P<0.05).

5. <u>The effect of peptide on the NK cell activity in BALB/c mice with transplanted ascitic fluid type liver cancer H_{22} (Method 4.3)</u>

Table IV.5. The effect of peptide on the NK cell cytotoxic activity index

Group	Dose	N	NK activity index(%)
CMS003	500μg/kg	17	37.9±14.5*^$

CMS014	0.5μg/kg	17	40.7±19.7$^{*\$}$
CMS024	0.5μg/kg	18	39.3±18.7$^{*\$}$
CMS024	5μg/kg	20	34.9±12.1$^{*\wedge\$}$
CMS024	50μg/kg	20	43.6±13.9$^{*\wedge\$\&}$
CMS032	5μg/kg	20	52.6±12.5$^{*\wedge\$\&}$
CMS032	50μg/kg	19	41.0±18.7$^{*\wedge\$\&}$
CMS034	50μg/kg	20	57.3±17.9$^{*\wedge\$\&}$
IL-2	3×10^{5}IU/kg	19	26.0±9.0
IFN-γ	3×10^{5}IU/kg	18	20.9±3.3
Cyclo-phos Phamide	20mg/kg	19	16.5±7.2*
Saline	0.5ml	20	24.0±8.2

*: as compared with saline, P<0.05.
^: as compared with IFN-γ, P<0.05.
&: as compared with IL-2, P<0.05.
$: as compared with cyclophosphamide, P<0.05

CMS003 at 500μg/kg/day, CMS014 at 0.5μg/kg/day, CMS024 at 0.5μg/kg/day, 5μg/kg/day, and 50μg/kg/day, CMS032 at 5μg/kg/day and 50μg/kg/day and CMS034 at 50μg/kg/day were found to be able to increase the NK cell cytotoxic activity in the testing animal model, having statistically significant difference from the saline group (P<0.05).

6. <u>The effect of peptide on the survival of DBA/2 mice with transplanted L_{1210} leukemia (Method 5)</u>

Table IV.6. The effect of peptide on the survival index of testing animals

Group	Dose	N	Survival days	Survival index (%)
CMS019	0.5μg/kg	20	21.1±5.8*	26.8
CMS035	0.5μg/kg	20	29.3±15.4*	76.1
IL-2	3×10^{5}IU/kg	20	32.0±13.7*	92.3
IFN-γ	3×10^{5}IU/kg	20	15.6±2.2	
Cyclophos	20mg/kg	20	24.0±5.3*	44.2

phamide			
Saline	0.5ml	21	16.6±5.6

*: comparing to saline group, P<0.05

CMS019 at 0.5μg/kg/day and CMS035 at 0.5μg/kg/day were found to be able to prolong the survival of DBA/2 mice with transplanted L1210 leukemia, having statistically significant difference from the saline group (P<0.05).

7. The effect of peptide on the humoral immunity of C_{57}BL/6 mice with transplanted B_{16} melanoma (Method 6.1)

Table IV.7. The effect of peptide on the hemolysis index of C57BL/6 mice with transplanted B16 melanoma

Group	Dose	N	Hemolysis index
CMS001	0.5μg/kg	20	51.0±16.2*$
CMS001	5μg/kg	20	41.3±17.7*$
CMS001	50μg/kg	19	45.0±31.9*$
CMS001	500μg/kg	20	36.0±10.2*$
CMS003	0.5μg/kg	20	61.6±26.9*$
CMS003	5μg/kg	20	37.2±15.9*$
CMS003	50μg/kg	20	38.7±13.5*$
CMS003	500μg/kg	20	35.9±13.0*$
CMS008	0.5μg/kg	19	42.7±18.4*$
CMS008	5μg/kg	20	38.9±12.0*$
CMS008	50μg/kg	20	37.1±16.7*$
CMS008	500μg/kg	20	50.1±17.8*$
CMS010	0.5μg/kg	18	34.9±10.5*$
CMS010	5μg/kg	20	51.0±14.6*$
CMS010	50μg/kg	20	39.6±7.7*$
CMS010	500μg/kg	20	50.1±16.7*$
CMS011	0.5μg/kg	20	32.0±14.7*

CMS011	500μg/kg	20	34.4±19.4*
CMS016	500μg/kg	20	43.3±29.9*
CMS019	0.5μg/kg	20	42.0±12.0*$
CMS019	5μg/kg	20	35.4±15.1*$
CMS019	50μg/kg	20	28.3±7.6*
CMS024	0.5μg/kg	20	43.0±10.7*$
CMS024	5μg/kg	20	42.2±11.8*$
CMS024	50μg/kg	20	27.8±9.1*
CMS024	500μg/kg	18	30.1±10.0*
CMS034	0.5μg/kg	18	50.8±18.4*$
CMS034	5μg/kg	19	43.0±11.7*$
CMS034	50μg/kg	20	30.2±10.9*
CMS035	5μg/kg	20	38.9±21.2*$
CMS035	50μg/kg	20	44.7±22.7*$
CMS035	500μg/kg	19	40.5±25.8*
rhIL-2	3×10^5IU/kg	19	49.3±24.7*
rmIFN-γ	3×10^5IU/kg	19	60.5±17.4*
Cyclo-phos phamide	20mg/kg	19	20.7±19.1
Saline	0.5ml	20	19.0±9.1

*: comparing to saline, P<0.05
^: comparing to rmIFN-γ, P<0.05
&: comparing to rhIL-2, P<0.05
$: comparing to cyclophosphamide, P<0.05

CMS001, CMS003, CMS008, CMS010, CMS011, CMS016, CMS019, CMS024, CMS034, and CMS035 were found to be able to enhance the humoral response (increase in hemolysis index) of C_{57}BL/6 mice with transplanted B_{16} melanoma at dosages as shown on Table IV.7, having statistically significant difference from the saline group (P<0.05).

8. <u>The effect of peptide on the survival of inoculated B16 melanoma cells in C_{57}BL/6 mice (Method 6.2)</u>

Postmortem examination of animals after the ending of test substance treatment did not show any sign of existence of B16 metastatic foci in the lung of mice treated with CMS008

at 0.5μg/kg/day, 5μg/kg/day, and 50μg/kg/day, and CMS016 at 5μg/kg/day and 500μg/kg/day.

CONCLUSION

In compliance with the Preclinical New Drug Research Guidelines issued by Branch of Drug Administration, Department of Health, PR China in 1993, the effects of peptide on mice with transplanted cancer cells was studied. It was concluded that

1. CMS010, CMS034, and CMS035 at suitable dosage could significantly inhibit the development of transplanted S_{180} sarcoma in mice;
2. CMS001 and CMS034 at suitable dosage could enhance the phagocytotic immune activities of mice transplanted with S_{180} sarcoma;
3. CMS008, CMS011, CMS024, and CMS032 at suitable dosage could prolong the survival of mice transplanted with ascitic fluid type liver cancer H22;
4. CMS010, CMS019, CMS024, CMS034, and CMS035 at suitable dosage could enhance T lymphocyte transformation in mice transplanted with ascitic fluid type liver cancer H22;
5. CMS003, CMS014, CMS024, CMS032, and CMS034 at suitable dosage could increase the NK cell cytotoxic activity of mice transplanted with ascitic fluid type liver cancer H22;
6. CMS019 and CMS035 at suitable dosage could prolong the survival of mice transplanted with L1210 leukemia;
7. CMS008 and CMS016 at suitable dosage could inhibit the development of transplanted B16 melanoma in mice;
8. CMS001, CMS003, CMS008, CMS010, CMS011, CMS016, CMS019, CMS024, CMS034, and CMS035 at suitable dosage could enhance the humoral immune response of mice transplanted with B16 melanoma.

Discussion

CMS001, CMS003, CMS008, CMS010, CMS011, CMS014, CMS016, CMS019, CMS024, CMS032, CMS034, CMS035 may be useful as a part, or on its own, in cancer management in human. For example, CMS001, CMS003, CMS008, CMS010, CMS011, CMS014, CMS016, CMS019, CMS024, CMS032, CMS034, and CMS035 may be used to increase the immunity of cancer patients. CMS008, CMS010, CMS016, CMS034, and CMS035 may be used to interfere with the growth of cancer cells in patients. CMS008, CMS011, CMS019, CMS024, CMS032, and CMS035 may be used to prolong the life expectancy of cancer patients. The peptides may be used on its own, in combinations of two

or more peptides, or in combination with other pharmaceuticals or food supplements as a total course for cancer management.

Table IV.8 Peptides Effective for Cancer

CMS code	SEQ ID No.
CMS001	1
CMS003	27
CMS008	3
CMS010	4
CMS011	30
CMS014	7
CMS016	9
CMS019	11
CMS024	16
CMS032	22
CMS034	24
CMS035	25

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V. THE EFFECT ON THE BODY WEIGHT

Healthy rats were fed with high nutrition diet for 5 weeks, with or without simultaneous peptide treatment (intramuscular 300μg/kg/day). Rats on an ordinary diet with saline injection were used as negative control. After 5 weeks of treatment, injection was stopped and the same diet was maintained for another three weeks. Body weight data was collected at once per week interval. Behaviour of the rats was also observed. Rats receiving peptide CMS015 were found to have statistically significant lower body weight increase compared with control during the course of peptide treatment. This trend of decreased body weight gradually reduced after ceasing the CMS015 treatment. It is concluded that CMS015 at suitable dosage level can reversibly control obesity development induced by over-feeding.

Materials

Sprague-Dawley (SD) rats, weighing 145±10g, were supplied by the Center of Experimental Animal of Guangzhou University of Traditional Chinese Medicine, PR China (certificate No.: 2000A019). Peptides were custom synthesized (of L-amino acids origin) by American Peptide Company, Inc., USA, and were diluted to 10μg/ml in normal saline. The high and normal nutrition diets were prepared in compliance with the guideline for pre-clinical research of anti-obesity drug, issued by SDA (State Drug Administration), PR China [1].

Methods

Healthy rats were randomized into the experiment, positive control, and negative control groups. 10 rats per group, half male and half female. The experiment group of rats was fed with high nutrition diet for 5 weeks with simultaneous intramuscular injection of 300μg/kg/day peptide, once per day. Positive control group received the same high nutrition diet but placebo saline injection, while negative control group, used to proof successful establishment of obesity model, received normal nutrition diet and placebo saline injection. After 5 weeks treatment, injection was stopped and the same diet was maintained for another 3 weeks. The rats were weighed at once per week interval. Behaviour of the rats was also observed.

Statistics

The data were presented as mean ± standard deviation. Paired *t* test or single-factor ANOVA was used for inter and intra group comparison. Statistical significance cut-off was at $P \leq 0.05$.

Results

1. Effect of peptide on the body weight of SD rat

Table V.1 Effect of peptide on the body weight of SD rats

	Positive Control Group (g)		CMS015 Treatment Group (g)	
	Male n=5	Female n=5	Male n=5	Female n=5
Pre-treatment	145.6 ± 13.6	133.6 ± 4.6	145.6 ± 8.5	129.2 ± 3.3
Week 1	194.4 ± 14.5	164.4 ± 8.7	183.8 ± 10.6	157.8 ± 8.3
Week 2	239.6 ± 13.4	188.0 ± 6.4	220.6 ± 12.2*	176.0 ± 11.2*
Week 3	265.0 ± 11.8	208.8 ± 8.2	239.0 ± 16.0*	196.0 ± 10.5*
Week 4	287.4 ± 17.7	227.2 ± 8.2	258.2 ± 18.1*	212.0 ± 13.5*
Week 5	299.4 ± 21.2	236.6 ± 10.9	268.8 ± 17.7*	221.4 ± 13.2*
Week 6	333.4 ± 27.1	249.4 ± 16.3	299.4 ± 21.2*	235.6 ± 16.3
Week 7	349.2 ± 28.9	261.2 ± 13.4	310.4 ± 25.9*	242.2 ± 18.8*
Week 8	374.4 ± 37.2	255.6 ± 11.5	337.4 ± 30.6	252.8 ± 22.5

In comparison with positive control group: * $p<0.05$

At the dose of 300 μg/kg/day, CMS015 was found to be able to limit the weight gain of obesity rats induced by over-feeding, having statistically significant difference from the control group ($p<0.05$). The difference of the treatment and control groups increased with the length of treatment. Upon termination of peptide CMS015 treatment in the treatment group, the difference of the treatment and control groups gradually reduced and became statistically insignificant after three weeks, showing that the effect of the peptide on SD rat body weight is reversible.

During the whole course of the experiment, it was observed that the appetite and activity of all groups of rats remained normal.

Discussion

It is concluded that CMS015 at suitable dosage level can limit the development of obesity induced by over-feeding. The peptide may put into human use for control of obesity. The peptides may be used on its own, in combinations of two or more peptides, or in combination with other pharmaceuticals or food supplements as a total course for obesity management.

In the present study, administration by intramuscular injection was tested, but this does not exclude the possible effectiveness of the peptide if administered via other alternative routes. The peptides may be administered via intravenous injection, intramuscular injection, intraperitoneal injection, subcutaneous injection, and subcutaneous implantation, with or without delivery facilitating device such as liposome, sustain release protection etc. The peptide may also be administered in any form of oral administration like tablet, capsule, suspension, solution etc, in the usual form without modification or in slow release form, or with or without gastro-enteric protection. The peptide may further be applied in any form of topic application like ointment, cream, gel etc with or without transdermal facilitating device, or as inhalant of powder, dissolved, or as liposome protected form. The peptide may also be interpreted into its genetic sequence and cloned into an expression system, on its own or in combination with other peptide sequences, to generate a resulting peptide molecule to make use of the activity of the peptide as described in this report, with or without purification of the resulting peptide.

Table V.2 Peptides Effective for Obesity

CMS code	SEQ ID No.
CMS015	8

References

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It is understood that it may be possible to add additional amino acids to the amino or carboxyl termini of the above disclosed peptides as another method of practising the present invention. For example, one or two amino acids may be added to the disclosed peptide without affecting its biological function. It may also be possible to add three or four amino acids and still maintain the function of the peptides. These are all referred to as variants of the same peptide. Alternatively, one or two amino acids may be deleted from the peptide without affecting its biological activity. It may further be possible for three or four amino acids to be deleted without affecting the biological function of the peptides. These are referred to as fragments of the instant peptide. Furthermore, derivatives of the peptide such as conservative replacement of one amino acid for another within the same functional class may be used to practise another aspect of the present invention. For example, peptides

having non-polar or hydrophobic side chains may be possible to substitute one side group for another without reducing biological activity. As a further example, linker/spacer may be inserted into the peptide to form variants, but the variants still retaining its active moiety as the original peptide used in this study These are also considered variants of the peptides. A peptide analogue as used herein, includes peptides that have amino acid molecules which mimic the structure of the natural amino acid e.g. an analog with a different backbone structure, or D-amino acid substitution. As a further example, although the amino acids used for synthesizing the peptides are in their L optical isomeric form, peptides with one or more of the amino acids in the sequence substituted with the D-form may have similar biological activities. The term "functional derivative" as used in the claims is meant to include fragments, variants, analogues or chemical derivatives of the peptide.

As used herein, the term "hybrid peptide" is used to refer to peptides that contain additional peptides inserted into the original biologically active peptides having SEQ. ID NOs. 1-30 or their functional derivatives, but still retain substantially similar activity. The additional peptides include leader peptides that contain, for example, an amino acid sequence that is recognized by one or more prokaryotic or eukaryotic cell as a signal for secretion of the hybrid protein into the exterior or the cell. The secretion may be a direct secretion, or indirectly through secretory vesicles.

"Substantially pure peptide" refers to peptides that are at least 10%w/w in purity, more preferably 20%, even more preferably 40% and much more preferably 60% and far more preferably larger than 90% pure. In the most preferred embodiment, the purity is larger than 99%. The substantially pure peptide can be used to prepare pharmaceutical and nutritional formulations that may be complex mixtures as described below.

The use of the above-identified peptides in pharmaceutical formulations may be employed as possible treatment for immunological disorders or disease having secondary effect on immunity e.g. cancer or infections or any of the conditions mentioned above. The formulations may contain one of identified peptides mixed with other active or inactive constituents, including other peptides. E.g. two to several (e.g. 3-5) of the listed peptides may be added to the same formulation with or without other ingredients. Alternatively, one of the listed peptides may be used to prepare the formulation together with peptides not listed here. They can be administered in the form of intravenous, intramuscular, intracutaneous, subcutaneous or intradermal. The mode of administration may also be intra-arterial injection that leads directly to the organ of problem. Other modes of administration are transdermal, inhalation as powder or spray, and other forms of delivery known by one in the art. The formulation may also be orally taken, and may contain carriers that can be used to prevent gastric digestion of the peptide after oral intake or any other carriers known in the art (for transdermal such as liposome).

The pharmaceutical formulation may include any of the known pharmaceutical carriers. Examples of suitable carriers include any of the standard pharmaceutically accepted carrier known to those skilled in the art. These include but are not limited to, physiological saline solution, water, emulsions including oil and water mixtures or triglyceride emulsions, and other types of agents, fillers, coated tablets and capsules. The appropriate carrier may be selected based on the mode of administration of the pharmaceutical composition.

The peptides may be administered via intravenous injection, intramuscular injection, intraperitoneal injection, subcutaneous injection, and subcutaneous implantation. The peptide may also be administered in any form of oral administration like tablet, capsule, suspension, solution etc, in the usual form without modification or in slow release form, or with or without gastro-enteric protection. The peptide may further be applied in any form of topic application like ointment, cream, gel etc with or without transdermal facilitating device. The peptide may also be interpreted into its genetic sequence and cloned into an expression system, on its own or in combination with other peptide sequences, to generate a resulting peptide molecule to make use of the activity of the peptide as described in this report.

The dose of each peptide may be 1ng – 10g per kg body weight. A preferred dose is 10ng - 10mg per kg, and more preferably 1μg - 1mg per kg for an injection mode of administration. However, the effective dose can be as low as 1ng per kg body weight, since one or more of the peptides may operate through receptors that will induce a cascade of normal physiological response. Alternatively, one or more of the peptides can just be an initiator for a whole cascade of reaction. For an oral intake, the amount may be 1ng - 10g per day per kg body weight, more preferably 0.1μg - 1g per day per kg body weight and even more preferably 1μg - 10mg per day.

VI. GENE THERAPY AND METHOD OF TREATMENT

Gene therapy based on the discovered peptide sequences is performed by designing a nucleic acid sequence that code for one of these peptides. The nucleic acid may be synthesized chemically and operably ligated to a promoter, and cloned into an expression vector. The expression vector is then administered into the human body as the form of gene therapy for expression in the human cell. The term "genetic vectors" as used herein includes these expression vectors. Vectors that can be used for gene therapy includes adeno-associated virus (Mizuno, M. et al. (1998). Jpn J Cancer Res 89, 76-80), LNSX vectors (Miller, A.D. et al. (1993) Methods Enzymol 217, 581-599) and lentivirus (Goldman, M.J. et al. (1997) Hum Gene Ther 8, 2261-2268).

Other vehicles for peptide delivery include expression vectors encoding the desired peptide that can be transferred into an organism which can replicate in the host organism to which it is desired to administer the peptide without significant detrimental effects on the

health of the host organism. For example, the expression vectors may be transferred into an organism which is not pathogenic to the host organism to which it is desired to administer the peptide. In some embodiments the expression vector produces the desired peptide in a bacterial or fungal organism which does not have significant detrimental effects on the health of the host organism to which the peptide is to be administered. For example, the expression vector encoding the desired peptide may be an expression vector which produces the desired peptide in an organism such as lactic acid bacteria, E. Coli, or yeast. In one embodiment, the expression vector produces the desired peptide in a microbe normally found in the mammalian gut or a microbe tolerated by the mammalian digestive tract. Some of the microbial species in which the desired peptide can be expressed include, but are not limited to, *Lactobacillus* species, such as *L. acidophilus, L. amylovorus, L. casei, L. crispatus, L. gallinarum, L. gasseri, L. johnsonii, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus* or others; *Bifidobacterium* species, such as *B. adolescentis, B. animalus, B. bifidum, B. breve, B. infantis, B. lactis, B. longum* or others; *Enterococcus faecalis* or *Ent. facium*; *Sporolactobacillus inulinus*; *Bacillus subtilis* or *Bacillus cereus*; *Escherichia coli*; *Propionibacterium freudenreichii*; or *Saccharomyces cerevisiae* or *Saccharomyces boulardii*

Nucleic acid sequences that encode any of the peptides of the present invention, chemically synthesized or produced by other means, including but not limited to the reverse transcription of mRNA to produce cDNA molecules, are incorporated into expression vectors for gene transfer into the desired organisms by methods of genetic engineering familiar to those of skill in the art. The expression vectors may be DNA vectors or RNA vectors. For example, the expression vectors may be based on plasmid or viral genetic elements. The expression vectors may be vectors which replicate extra-chromosomally or vectors which integrate into the chromosome.

The expression vectors comprise a promoter operably linked to a nucleic acid encoding a peptide of the present invention. The promoter may be a regulatable promoter, such as an inducible promoter, or a constitutive promoter. In some embodiments, the promoter may be selected to provide a desired level of peptide expression. In addition, if desired, the expression vectors may comprise other sequences to promote the production, presentation and/or secretion of peptides. In some embodiments a nucleic acid encoding a peptide of the present invention is operably linked to a nucleic acid sequence which directs the secretion of the peptide. For example, the nucleic acid encoding the peptide of the present invention may be operably linked to a nucleic acid encoding a signal peptide.

In some embodiments, the expression vectors which are engineered to encode the peptides of the present invention may be expression vectors which are adapted for expressing the peptide of the present invention in a bacterial species that makes up the normal gut flora of mammals, such as *Lactobacillus* species and *Bacillus subtilis* Examples of such expression vectors can be found in US Patents No. 6,100,388, to Casas, and No.

5,728,571, to Bellini, respectively. These documents are hereby expressly incorporated by reference in their entireties. It will be appreciated that any expression vector which facilitates the expression of a peptide of the present invention in an organism which is not detrimental to the health of the host organism to which the peptide is to be administered may be used.

In some embodiments, the expression vectors which are engineered to encode the peptides of the present invention may be expression vectors which are adapted for expressing the peptide of the present invention in a yeast species that is well tolerated by the mammalian gut , such as *Saccharomyces cerevisiae;* or, preferably, *Saccharomyces boulardii*, which can colonize the human gut and is used to treat certain forms of diarrhea. Yeast expression vectors can be used that constitutively express heterologous proteins and peptides, are highly stable, thus are well transmitted to progeny cells during mitosis and meiosis and may comprise coding sequence for a signal peptide or peptides that direct high levels of recombinant protein secretion. An example of such a yeast vector is given in US Patent No. 6,391,585, to Jang *et al.*, which is hereby expressly incorporated by reference in its entirety.

The expression vectors encoding the peptides of the present invention may be introduced into the organism in which it is intended to express the peptides through techniques known in the art. These techniques include traditional methods of transforming bacteria, yeast, or other microbes, through the use of chemically competent bacterial cells, electroporation or lithium acetate transformation (for yeast), for example, as well as recent advances in the transformation of bacterial species recalcitrant to these procedures. In some embodiments, the expression vectors are introduced into lactic acid bacteria known to be recalcitrant to transformation using the method disclosed by Leer *et al.* (WO 95/35389), the disclosure of which is incorporated herein by reference in its entirety. The introduced sequences may be incorporated into microbial chromosomal DNA or may remain as extrachromosomal DNA elements.

This genetically engineered microbe containing the expression vector can then be inoculated into the alimentary canal, vagina, trachea etc. to achieve sustained immuno-therapy. In some embodiments, the organisms expressing the peptides of the present invention are ingested in an inactive form or, preferably, in live form. In the gut these microorganisms produce said peptides, release them into the lumen by secretion or by lysis of the microorganism or otherwise present the peptides to the host, whereby the peptides produce their intended effect upon the host organism. In other embodiments, peptides are presented to the host at the mucous membrane of the nasal passages, vagina or the small intestine.

Another method of the treatment is the use of liposomes as a means for delivering the specific nucleic acid to the cells in the human body. The nucleic acid (such as an expression vector containing a nucleic sequence that encodes peptides of sequence ID No.1 to ID No.30) is delivered in an environment that encourages cellular uptake and chromosomal incorporation as described in Gao, X. and Huang, L. (1995) Gene Ther 2, 710-722 and US 6,207,456. Alternatively, the peptide itself can be encapsulated in the liposome and delivered directly, using a method described in US6,245,427. All the scientific publications and patents indicated above are incorporated herein by reference in their entireties.

The nucleic acid sequences useful for the above-mentioned gene therapy and method of treatment include sequences that code for these peptides and functional derivatives thereof. Any one of the numerous nucleic acid sequences may be used to code for these peptides and their derivatives based on the degenerate codon system.

The following references are incorporated herein by reference in their entireties.

Principles of Pre-clinical Research of New Drugs, People's Republic of China. 1993, 7:134-135Shuyun Xu, Rulian Bian, Xiu Chen. Methodology of pharmacological experiment. People's Health Publishing House. 1991, 1221-1234

1. Principle of new drug research in pre-clinic issued by Ministry of Health, People's Republic of China. 1993, 7:140
2. Jinsheng He, Ruizhu Li, Tingyi Zong. The study on MTT reduction method of testing NK cell activity. China Immunology Journal. 1996, 1(6): 356-358
3. Qian Wang. Modern medical experiment method. People's Health Publishing House. 1998, 482-483
4. Principle of new drug research in pre-clinic issued by Ministry of Health, People's Republic of China. 1993, 7: 141
5. Principle of new drug research in pre-clinic issued by Ministry of Health, People's Republic of China. 1993, 7: 132-133
6. Principle of new drug research in pre-clinic issued by Ministry of Health, People's Republic of China. 1993, 7: 128-129
7. Yuanpei Zhang, Huaide Su. Phamalogical experiment (second edition). People's Health Publishing House. 1998, 137-138
8. Jiatai Li, clinical pharmacology(second edition). People's Health Publishing House. 1998, 1338-1339.

VII. PEPTIDE CONJUGATIONS TO AND FORMULATIONS WITH BIOLOGICALLY USEFUL MOLECULES

The biologically active peptides of the present invention may be conjugated to other biologically effective or useful molecules to provide an additional effect or use or to enhance their therapeutic effectiveness. Many potential conjugating molecules, their biological effects and the methods for conjugation of the molecules to peptides are known in the art. For other candidate conjugation partners, chemical reactions for conjugating the instant peptides thereto can be deduced by one skilled in the art without undue experimentation. Effective molecules are described below. Specific examples of how various peptides according to the present invention may be conjugated to their effective molecules and the biological properties of the resulting conjugation product are described. It is understood that other peptides of the instant invention may also be conjugated in similar reactions.

Some of the peptides of the invention have distinct therapeutic effects on a particular cell or tissue type. One important objective of conjugating molecules to peptide drugs is the targeting of the peptide to a particular location or compartment within the body of an individual being treated. In this way, the peptide drug and its effects can be concentrated at the location of the cell or tissue type on which it has the intended therapeutic effect. This can augment the effect that a similar molar amount of the free, unconjugated peptide would have. Conversely, the dosage of a conjugated peptide drug that is targeted to its therapeutic active site can be significantly lower than the dosage required to get the same therapeutic effect from the free, unconjugated form of the drug. For example, the conjugation of the peptide SEQ ID No. 30 (CMS30), which is indicated for nephritis, to the low-molecular-weight protein (LMWP) lysozyme would lead to the preferential uptake of the peptide by the proximal tubules of the kidney (Haas *et al.*, Kidney Intl.,52(6):1693, 1997). This will enhance the effectiveness of the peptide by concentrating it in the target area of its anti-nephritic activity.

Another beneficial effect of targeting a peptide drug to the site where its activity is most desired is the reduction of unwanted side effects. A peptide drug that is administered in order to effect a change in a particular cell or tissue type can also act in other locations within an individual, sometimes with detrimental results. By targeting the peptide to the desired location of activity via conjugation to a targeting molecule, the concentration of peptide elsewhere in the individual and the subsequent side effects can be reduced. In the nephritis treatment with CMS30 discussed in the preceding paragraph, the preferential uptake of the LMWP-CMS30 conjugate by the kidney would lead to decreased availability of the conjugate elsewhere. Any undesired effects caused by CMS30 activity on tissues or cells outside of the kidney will then be reduced.

Peptides comprising consisting essentially of or consisting of one of SEQ ID NOs.1-30 or functional derivatives thereof can be conjugated to a variety of molecules for targeting to different locations throughout the body of an individual. Any of the conjugation technologies described below for targeting a peptide to a desired location, as well as other conjugation technologies familiar to those skilled in the art, may be employed with any of the peptides of the present invention. For example, the selective delivery of an anti-hepatitis B drug to liver cells has been demonstrated (Fiume *et al.*, Ital J Gastroenterol Hepatol, 29(3):275, 1997, which is incorporated herein by reference in its entirety). In this study, researchers conjugated adenine arabinoside monophosphate (ara-AMP), a phosphorylated nucleoside analogue active against hepatitis B virus, to lactosaminated human albumin, a galactosyl-terminating macromolecule. Hepatocytes express a receptor protein that interacts with terminal galactosyl residues with high affinity. Through binding to this receptor, the conjugated drug will be selectively taken up by hepatocytes. After absorption, the conjugated drug is delivered to lysosomes, where the bond between the two components of the conjugated drug is cleaved, releasing ara-AMP in its active form. In the study cited above, the conjugated drug was as effective as free ara-AMP in treating patients with chronic hepatitis B infections, but did not cause the clinical side effects, such as neurotoxicity, that the administration of free ara-AMP causes. Such an approach can be used with any of the peptides of the present invention. A peptide that is indicated for the treatment of hepatitis B, such as CMS001, can be conjugated to lactosaminated human albumin or another galactosyl-terminating macromolecule. Like the nucleoside compound above, CMS001 will then be preferentially taken up by hepatocytes and concentrated in the tissues that are infected with the hepatitis B virus.

In a related study to the one above, by the same research team (Di Stefano *et al.*, Biochem. Pharmacol., 61(4):459, 2001), an anti-cancer chemotherapeutic agent, 5-fluoro 2-deoxyuridine (FUdR), was conjugated to lactosaminated poly-L-lysine in order to target the compound to the liver and treat liver micrometastases. The drug is selectively taken up by liver cells, which cleave the bond between FUdR and the targeting molecule. A portion of the free FUdR will then exit the liver cells and a localized therapeutic concentration of the anti-cancer agent is created. This concentration is sufficient for pharmacological activity on the metastatic cells that have infiltrated the liver. Because the drug is selectively concentrated in the liver, the dosage of the conjugated drug can be significantly less than the smallest pharmacologically active dosage of the free, unconjugated compound. This strategy can be utilized with any of the peptides of the present invention. For instance, conjugation of lactosaminated poly-L-lysine to the anti-HBV peptide CMS001 could significantly reduce the dosage of CMS001 necessary to treat a hepatitis B infection in an individual.

The targeting of compounds to particular tissues or cell types within the body has been achieved for a number of different tissues or cell types. For example, tumor cells often

express abnormally high levels of peptide hormone receptors on their surfaces, such as bombesin, lutenizing hormone-releasing hormone, and somatostatin. In one study, the anti-cancer compound paclitaxel (taxol) has been selectively targeted to hormone-secreting tumor cells that express somatostatin receptors at a high density by conjugating the drug with octreotide, an analog of somatostatin. The ostreotide-conjugated taxol was just as effective as free taxol but with reduced toxicity to normal cells (Huang *et al.*, Chem. Biol., 7(7):453, 2000). Several peptides of the present invention, such as CMS024 and CMS034, have shown potent anti-tumor activity in animal studies. Using the techniques of Huang *et al.* to conjugate these peptides to octreotide would create a potential anti-cancer treatment specifically targeting tumor cells expressing high levels of somatostatin. This approach can be adapted to target tumor cells overexpressing any number of peptide hormone receptors. In another example of targeting a drug to a specific tissue type, poly (L-aspartic acid) was used as a carrier molecule to target drug delivery to colon cells specifically (Leopold *et al.*, J. Pharmacokinet. Biopharm., 23(4):397, 1995).

Beyond the specific targeting of a peptide drug to a particular cell or tissue type, conjugation of peptides comprising, consisting essentially of, or consisting of one of SEQ ID NOs. 1-30 or a functional derivative thereof to carrier molecules can provide other ways to enhance the delivery of peptide drugs, thereby augmenting or otherwise improving their therapeutic effects. Any of the conjugation technologies described below may be used with any of the peptides of the present invention, as with other technologies familiar to those skilled in the art. The effectiveness of any drug will be hampered if the compound cannot be delivered to its target efficiently. A drug must be transported, actively or otherwise, to the site of its activity without substantial loss of activity due to metabolic processing or degradation. Peptide drugs are subject to the activity of peptidases and, as highly charged molecules, can be refractory to transport across lipid cell membranes and endothelial cell membranes, such as the blood-brain barrier. Conjugation to other molecules provides a way to protect peptides from degradation and to enhance the absorption of peptide drugs into cells or anatomical compartments that would normally exclude the compounds.

By allowing peptides access to locations within the body from which they would normally be excluded, conjugation techniques can open up new routes for administration of the drug. In Patel *et al.*, Bioconjugate Chem., 8(3):434, 1997, the chemistry of which is detailed in Example 5 below and which is incorporated herein by reference in its entirety, researchers conjugated a peptide drug known to be a potent analgesic, the heptapeptide deltorphin, to an organic molecule that was specifically designed to allow the peptide to cross the blood-brain barrier. This allows the drug to be administered intravenously instead of by intracerebro ventricular injection.

The carrier molecule in Patel *et al.* was designed to specifically target those endothelial cells that comprise the blood-brain barrier in addition to allowing the peptide to get across the barrier. Endothelial cell membranes throughout the body, including the blood

brain barrier, are heterogeneous with regards to the sequence specificity and concentration of membrane-bound endopeptidases that are displayed on their surfaces. The design of the molecule exploits this characteristic to enable targeting of the carrier molecule and its cargo. The molecule contains three fatty acid chains whose free ends are capped with the dipeptide Arg-Pro, which will interact preferentially with the endopeptidases of the blood brain barrier. The transport of the charged peptide drug molecule is then enabled by the lipophilic fatty acids chains. Thus the dipeptide-capped triglyceride molecule permits both the targeting and the transport across the blood brain barrier.

Conjugation methods can also enhance the kinetics of a peptide drug's activity. Any of the conjugation technologies described below for enhancing the kinetics of a peptide's activity as well as other conjugation technologies familiar to those skilled in the art may be employed with a peptide comprising, consisting essentially of or consisting of one of SEQ ID NOs. 1-30 or a functional derivative. Patel *et al.* found that the conjugated form of the analgesic peptide was not only able to enter the brain from the bloodstream, but had sustained action in comparison to the free peptide as well. The intravenously administered drug took longer to have a therapeutic effect, but the effect lasted longer and decreased more slowly than the effect of the free peptide injected intracranially. The researchers found that the conjugated peptide molecule is remarkably stable in serum, yet had no effect when injected intracerebro ventricularly, indicating that the carrier molecule is likely degraded and removed during its transport from the bloodstream to the brain. They suspect that the time required to transport the conjugate and degrade the carrier molecule is the cause of the altered kinetics. Regardless of the mechanics of the delay, in a clinical setting, the intravenous stability of the conjugated peptide molecule and the prolonged onset and activity of the drug's effects would mean that it could be administered less frequently. A less frequent and thus more convenient dosing schedule enhances the practical value of the drug as a treatment option.

As would be apparent to a person of skill in the art, the techniques and procedures of Patel *et al.* are readily adaptable to the delivery of any peptides that fall within a limited size range, including any of the peptides of the present invention. For example, a peptide of the present invention that exhibits an anti-cancer effect, such as CMS024, could be conjugated to the same molecule used by Patel *et al.*. In the treatment of an individual with brain cancer, the conjugated molecule would allow CMS024 access to the brain from the bloodstream and allow CMS024 to exert its effects on the tumor tissue in the brain. Modifications to alter the targeting of the carrier molecule would also be apparent to such a person. The targeting feature of the carrier molecule is a function of the identity of the two amino acids that comprise the dipeptide mask at the end of the fatty acid chains. The Arg-Pro dipeptide interacts preferentially with the set of membrane-bound endopeptidases found on the surface of the blood brain barrier's endothelial membrane. Other endothelial cells and membranes could potentially be targeted by other dipeptide combinations.

Conjugation has also been used by researchers to create peptide drugs that can be effectively absorbed through the digestive tract or transdermally. Any of the conjugation technologies for enhancing absorption described below, as well as other conjugation technologies familiar to those skilled in the art, may be used to enhance the absorption of a peptide comprising, consisting essentially of or consisting of one of SEQ ID NOs. 1-30 or a functional derivative thereof. Kramer *et al.* describe a procedure for the coupling of peptide drugs to bile acids. The absorption rate for the conjugated molecule following oral delivery of the compound is significantly enhanced as compared to the peptide alone (J. Biol. Chem., 269(14): 10621, 1994). Toth *et al.* (J.Med.Chem., 42(19):4010, 1999) describe the conjugation of a peptide drug with anti-tumor properties to lipoamino acids (LAA) or liposaccharides (LS), in order to increase the absorption rate and enhance the delivery of the anti-cancer peptide to its active site. In their study, a derivative of somatostatin that shows strong anti-proliferative properties, but has impaired pharmokinetics, is conjugated to either LAA or LS. The resulting conjugate drug has improved absorption profiles across skin and gut epithelium and increased resistance to degradation while still active against tumor cells. These techniques would be very useful in conjunction with any of the peptides of the present invention. CMS015 has displayed an anti-obesity effect in mice, where it was delivered via intramuscular injection. However, by exploiting the techniques utilized in the examples above, a conjugated form of CMS015 could be developed that would have a therapeutically sufficient bioavailability when delivered to an individual orally. By increasing the rate of absorption of the molecule across the intestinal epithelium, more of the peptide can be delivered to the bloodstream and exert its effect on the individual being treated.

Conjugation may also be used to provide sustained release of a peptide drug. Any of the conjugation technologies for providing sustained release, as well as other conjugation technologies familiar to those skilled in the art, may be used to provide sustained release of a peptide comprising, consisting essentially of or consisting of one of the SEQ ID NOs. 1-30 or a functional derivative thereof. As seen above in the work of Patel *et al.*, the sustained delivery of a peptide drug can be achieved with conjugation methods. Another example is the work of Kim *et al.* (Biomaterials, 23:2311, 2002), where recombinant human epidermal growth factor (rhEGF) was conjugated to polyethylene glycol (PEG) before microencapsulation in biodegradable poly(lactic-co-glycolic acid) (PLGA) microspheres. Microencapsulation in PLGA has been used by several groups to deliver various growth factors and morphogenic proteins (Meinel *et al.*, J. Controlled Rel., 70:193, 2001). Through conjugation to PEG, rhEGF became resistant to forming water-insoluble aggregates and to adsorption to the water-organic phase interface during micelle formation with PLGA as compared to unconjugated, free rhEGF. The pharmokinetics of the formulation with the conjugated hormone were improved, showing longer lasting, steadier and overall greater drug activity than with the free hormone, which the researchers speculate is due to the enhanced physical stability of the hormone conjugated to PEG. A similar strategy could be

employed to create sustained release formulations of any of the peptides of the present invention. For example, CMS010 has potent stimulatory effects on cells of the immune system. By conjugating PEG to this peptide and incorporating the conjugated drug into PLGA microspheres, the stimulatory effects of CMS010 on an individual can be longer lasting and more stable, as the dosing of the drug, as it is being released from its PEG conjugate, is more even and ensures a more constant delivery of the peptide drug to the immune system.

Prolonged release of a peptide drug can significantly enhance its activity. Any of the conjugation technologies for providing prolonged release of a peptide described below, as well as other conjugation technologies familiar to those skilled in the art, may be used to provide prolonged release of a peptide comprising, consisting essentially of or consisting of one of the SEQ ID NOs. 1-30 of a functional derivative thereof. Oldham *et al.* (Int. J. Oncology, 16:125, 2000) compares the anticancer agent paclitaxel against a new form of the drug, paxlitaxel conjugated to poly(L-glutamic acid) (PG-TXL). PG-TXL appeared to have superior anti-tumor activity compared to free paclitaxel, suggesting that the drug has superior pharmokinetic properties or maybe even a superior method of action. However, investigators found that PG-TXL exerted its effects by the same mechanism of action as the free drug, inducing cell cycle arrest by disturbing the polymerization of microtubules subunits. Evidence suggests that the superior anti-tumor activity of the conjugated drug arises from a continuous and steady release of the free drug from the conjugate, maintaining its therapeutic concentration for a longer period as compared to administration of the free peptide. The addition of poly(L-glutamic acid) tail to a peptide of the invention with anti-cancer properties, such as CMS008, could enhance its tumor-killing ability as well.

The enzymatic degradation of peptides represents a serious barrier to effective use of peptides as drugs. Any of the conjugation technologies for reducing enzymatic degradation of a peptide described below, as well as other conjugation technologies familiar to those skilled in the art, may be used to reduce the enzymatic degradation of a peptide comprising, consisting essentially of or consisting of SEQ ID NOs. 1-30 or a functional derivative thereof. Researchers have developed numerous approaches to protect peptides from luminally secreted proteases in the gut as well as membrane-bound peptidases. The latter are found on the surface of all mucosal tissues, the crossing of which is often the route of entry for peptide drugs. Bernkop-Schurch *et al.* (J. Drug Target., 7:55, 1999) report the creation of peptide drug formulations containing inhibitors of pepsin. An analogue of pepstatin was covalently attached to mucoadhesive polymers; this novel pepsin inhibitor was included in tablets containing insulin. After incubation under laboratory conditions simulating digestion, all of the insulin from control tablets was metabolised, whereas nearly 50% of the insulin from tablets containing the inhibitor was protected from degradation. In another study, the same group utilized protease inhibitors at dosages that would normally cause toxic side effects to inhibit degradation of biologically active peptides (Bernkop-

Schnurch *et al.*, Adv. Drug Del. Rev., 52:127, 2001). This approach utilizes chitosan, an aminopolysaccharide related to cellulose that is extracted from chitin, a major structural polysaccaride found in crustaceans and other organisms. By conjugating the protease inhibitors to chitosan and including this conjugated molecule in the formulation of the peptide drug, significant inhibition of digestive tract proteases was seen, increasing the bioavailability of the peptide, without the side effects that would be expected with administration of free protease inhibitors. In the study, a variety of protease inhibitors alone and in combination were utilized for conjugation to the chitosan carrier. A chitosan-EDTA conjugate inhibited endogenous proteases as well, by binding mineral co-factors required by certain proteases for activity. As would be readily apparent to one with skill in the art, a large number of possible combinations between carrier molecules and effector moieties could be created to provide beneficial properties to peptide formulations, any of which could easily be adapted for use with a peptide of the present invention. For example, these techniques could be used with the anti-obesity peptide CMS015. By creating a formulation for oral delivery of the peptide using protease inhibitors bound to chitosan, oral delivery of CMS015 could be used in place of intramuscular injections. This approach does not rule out using the more absorbable, conjugated version of CMS015 (discussed in a paragraph above) in this formulation, to create an even greater level of bioavailability for this peptide.

In addition to being targeted to a location by another molecule, peptides themselves can serve as the molecule that targets. Any of the conjugation technologies for using a peptide to target a molecule to a desired location described below, as well as other conjugation technologies familiar to those skilled in the art, may be used with a peptide comprising, consisting essentially of or consisting of one of SEQ ID NOs. 1-30 or a functional derivatve thereof. For example, researchers have taken the anticancer drug difluoromethylornithine (DFMO) and conjugated it to a peptide for targeting purposes. DFMO is a highly cytotoxic agent that is effective in killing a variety of tumor cell types. However, since it is rapidly cleared from the body, its therapeutic value is limited. In this study, DFMO has been conjugated to a particular fragment of α melanotropin and an analogue of the fragment containing two amino acid substitutions that was shown to bind preferentially to the melanotropin receptors on a human melanoma cell line (Suli-Vargha *et al.*, J. Pharm. Sci., 86:997, 1997). To facilitate the liberation of DFMO from the peptide fragments by aminopeptidases, the drug was conjugated to the N-terminal ends of the peptides. The researchers found that the conjugated drugs are more effective at killing melanoma cells that the unconjugated drug alone.

The effects of the peptides of the present invention may be due in part to a targeting ability inherent in the peptides themselves. For instance, like the α melanotropin fragment, a particular peptide of the invention may bind to a certain receptor found on the surface of a distinct type of cell. By using that peptide as a conjugant, a drug could be targeted to the location of those cells within the body of an individual being treated with the drug.

Peptides as conjugates can serve functions other than targeting. Any of the conjugation technologies for enhancing the therapeutic effectiveness of a peptide described below, as well as other conjugation technologies familiar to those skilled in the art, may be used to enhance the therapeutic effectiveness of a peptide comprising, consisting essentially of or consisting of one of SEQ ID NOs. 1-30 or a functional derivative thereof. Fitzpatrick *et al.* have improved upon a conjugated anticancer agent by using a peptide spacer between the two molecules (Anticancer Drug Design, 10:1, 1995). Methotrexate had already been conjugated to human serum albumen (HSA) to increase its uptake by and activity against tumor cells. Once taken up by a cell, some of the methotrexate is liberated from the conjugate by enzymes in the lysosome and can then exert its cytotoxic effects. By inserting a four amino acid linker peptide between the methotrexate and the HSA that is easily digested by lysosomal enzymes, the amount of active methotrexate generated within cells from the conjugate molecule was increased. The peptides of the present invention may be exerting their effects through specific interaction with particular enzymes. By incorporating a peptide of the invention into a conjugated molecule as a linker segment between a drug and its carrier molecule, or in addition to another linker segment, the pharmacokinetics can be altered. This can create a pro-drug that is more resistant or more susceptible to the activity of proteases and subsequently increase or decreasing the rate of drug molecule release from the conjugate. As seen in the examples of conjugated chemotherapy agents above, altering that rate of drug molecule delivery can greatly enhance the effectiveness of a drug.

The effects of a drug on a particular cell may be altered depending upon other factors such as the activation state of a cell or the presence of other molecular signals near or within the cell. In some cases, in order for a drug to have an effect, another molecule or signal needs to be present. Damjancic *et al.* (Exp. Clin. Endocrin., 95:315, 1990) studied the effects of human atrial natriuretic peptide (hANP) on patients with deficient endogenous glucocorticoid synthesis. The peptide was given to patients during a withdrawal of glucocorticoid therapy or during subsequent resumption of therapy using dexamethasone. Patients responded to hANP with an increase in diuresis and sodium excretion only when the peptide hormone was given during concomitant dexamethasone treatment. Treatment with hANP during withdrawal of glucocorticoid therapy had no effect. The effect of concurrent steroid hormone administration can also be to enhance the activity of a peptide. In a report from Zhu *et al.* (Acta Pharm. Sinica, 28:166, 1993), the activity of the analgesic peptide kyotorphin (KTP) was significantly enhanced by conjugation to hydrocortisone via a short linker segment, as compared to the action of the peptide alone. No effect was seen with the administration of hydrocortisone alone.

The results of these studies illustrate the ability of steroid hormones as conjugated molecules or as ingredients in formulations can allow or enhance the activity of biologically active peptides. Any of the peptides of the present invention may also be modulated or

activated by conjugation to or co-application of steroid hormones. The techniques of Zhu *et al.* can be readily adapted for conjugation of steroid molecules to peptide of the present invention. Figures 1 through 5 also provide exemplary step-wise synthesis reactions for linking steroid hormones to any of the peptides of the present invention.

The examples presented above provide exemplary ways to augment the usefulness and the activities of any of the peptides of the invention. Further developments in this field will help overcome the barriers to creating effective peptide-based clinical treatments. As would be apparent to one with skill in the art, the techniques, reagents and protocols developed for use in peptide biochemistry, pharmaceutical research and clinical testing are all readily appliable to any of the peptides of the present invention.

EXAMPLE 1

Delivery of peptides through genetically engineered *Lactobacillus* bacterial species

The following is provided as one exemplary method to deliver peptides of this invention to a host as described above. A DNA sequence that encodes one of the peptides listed in table A above is synthesized by chemical means and this DNA sequence is inserted into an expression vector using standard techniques of genetic engineering familiar to those skilled in the art. The expression vector selected contains a constitutive promoter functional in *Lactobacilli,* a multiple cloning site for the introduction of DNA sequences in a specific 5' to 3' orientation as well as a selectable marker gene that confers resistance to an antibiotic (to aid in cloning procedures) and may comprise other sequences to assist in the production and/or secretion of the peptides, such as signal peptide sequences. An example of such a vector is provided by US Patent No. 5,592,908, to Pavla, which is incorporated herein by reference in its entirety. Briefly, this patent discusses several known promoters that function in *Lactobacillus* species, as well as a method for discovering novel promoters in said bacteria, any of which may be operably linked to a nucleic acid encoding a peptide of the present invention to express the peptide in *Lactobacilli.* A nucleic acid encoding a signal peptide, such as peptides comprising of 16 to 35 mostly hydrophobic amino acids that are active in *Lactobacillus lactis* described in US Patent No. 5,529,908, cited above, is interposed between the promoter and the nucleic acid encoding the peptide of the present invention such that the nucleic acid encoding the signal peptide is in frame with the nucleic acid encoding the peptide of the present invention.

In addition to the coding sequence of the peptide, the DNA sequence synthesized may comprise sequences to aid in the ligation and cloning of said DNA into the expression vector. For example, restriction enzyme recognition sites that correspond to ones found in the multiple cloning site of the vector can be incorporated into the synthesized DNA at the 5' and 3' ends of the sequence, so that the sequence can be cloned in proper orientation within the vector. Both the vector and the synthesized DNA are digested with the particular

restriction enzymes, then purified. Ligation reactions with the vector and the synthesized DNA are followed by transformation into a suitable strain of *E. Coli*. The transformed bacteria are plated on media containing the antibiotic to which the vector confers resistance. A colony of transformed bacteria is selected for growth cultures and plasmid preparation procedures; the presence of the synthesized DNA in the correct orientation is confirmed.

This expression vector is then transformed into a bacterial host cell of a *Lactobacillus* species, such as *L. acidophilus*. Transformed cells are selected for by virtue of the selectable marker found within the vector sequence and the secretion of the peptide may be verified by performing a western blot, performing gel electrophoresis of peptides present in the growth medium or other standard techniques. A transformed colony of bacteria is chosen and used to prepare large-scale cultures of the genetically engineered bacteria. A culture of the genetically engineered bacteria expressing the desired peptide is grown up and at least a portion thereof is administered to the alimentary canal, vagina, trachea or other area of the host organism in which the bacteria are able to replicate. If desired, the bacterial cultures can be treated in a variety of ways to produce a supplement for enteric consumption by the host. These treatments include lyophilization or other methods of preserving the bacteria, in addition to combining the bacteria with carrier agents, such as solutions, solvents, dispersion media, delay agents, emulsions and the like. The use of these agents to prepare supplements is well known in the art. For example, the bacteria can be used to make cultured milk products or other foodstuffs for human consumption, such that the organism expressing the peptide colonizes the gut of the host organism. A number of different methods for incorporating specific strains of lactic acid bacteria into foodstuffs such as yogurt, kimchee, cheese and butter are disclosed in US Patent No. 6,036,952, to Oh, which is incorporated herein by reference in its entirety. Upon consuming the bacteria through one of any number of routes, the engineered organisms can colonize the gut and allow the presentation and/or absorption of the peptides of this invention via the mucosal layer of the gut.

EXAMPLE 2

Delivery of peptides through a genetically engineered form of *Bacillus subtilis*

The following is provided as another exemplary method to deliver peptides of this invention to a host as described above. A DNA sequence that encodes one of the peptides listed in table A above is synthesized by chemical means and this DNA sequence is inserted into an expression vector via techniques of genetic engineering, all techniques being known in the art. The expression vector selected comprises a shuttle vector, such as pTZ18R (Pharmacia, Piscataway, NJ), capable of being propagated in both *E. Coli* and *B. Subtilis* and containing an antibiotic resistance gene for selecting colonies of transformed bacteria. This vector can contain a constitutive promoter active in *B. subtilis*, such as a promoter derived from the Sac B gene of *B. subtilis* as well as a nucleotide sequence encoding a signal peptide

active in *B. subtilis* that directs efficient export of expressed heterologous proteins from the bacterial cell. An example of such a vector is disclosed in US Patent No. 6,268,169, to Fahnestock, the disclosure of which is incorporated herein by reference in its entirety. Briefly, as detailed above, the DNA encoding a peptide of this invention will be synthesized with restriction enzymes sites and/or other sequences to facilitate cloning of the DNA through techniques familiar to those with skill in the art. After transformation into *E. Coli.*, plating, selection and propagation of the plasmid to create a plasmid stock, the plasmid is then be transformed into *B. subtilis* and transformants are selected by virtue of resistance to an antibiotic in the plating media.

Peptide production in and secretion from the genetically engineered *B. subtilis* is verified using techniques well known to those with skill in the art, such as radiolabeling of peptides for autoradiographic detection after SDS-PAGE anaylsis or Western blotting.

A culture of genetically engineered bacteria is grown up and at least a portion thereof is administered to the alimentary canal, vagina, trachea or other area of the host organism in which the bacteria are able to replicate.

EXAMPLE 3

Delivery of peptides through genetically engineered *Saccharomyces* yeast species

The following is provided as another exemplary method to deliver peptides of this invention to a host as described above. A DNA sequence that encodes one of the peptides listed in table A above is synthesized by chemical means and this DNA sequence is inserted into an expression vector via techniques of genetic engineering, all techniques being known in the art. The expression vector selected comprises a stably maintained yeast protein expression vector, comprising a constitutive yeast promoter such as pADH1, sites for replication of the vector in both yeast and *E. Coli*, a gene or genes that confer prototrophy to an auxotrophic yeast mutant for selection purposes, a multiple cloning site (MCS) and, if desired, sequences that code for a signal peptide. Vectors such as this are commercially available and well known in the art or can be readily constructed using standard techniques After insertion of the synthesized DNA into the yeast vector, transformation into *E. Coli*, plating of transformed *E. Coli* onto selective media, selection of a transformed bacterial colony and preparation of plasmid DNA from a growth culture of bacteria from said colony, the vector is transformed into *Saccharomyces cerevisiae* via well-known techniques such as lithium acetate transformation or electroporation. The strain of *Saccharomyces cerevisiae* selected for transformation is a mutant auxotrophic strain that will require a gene on the plasmid in order to grow on minimal media plates. Transformed yeast colonies are isolated by plating the yeast on growth media lacking the gene provided on the vector. Only those yeast that have received the vector and its selective gene and are expressing that gene product will be able to grow into colonies on the minimal media. Verification of peptide

secretion can be obtained by performing a Western blot, performing gel electrophoresis of peptides present in the growth medium or other standard techniques.

A transformed colony of yeast is chosen and used to prepare large scale cultures. A culture of the genetically engineered yeast expressing the desired peptide is grown up and at least a portion thereof is administered to the alimentary canal, vagina, trachea or other area of the host organism in which the bacteria are able to replicate. If desired, the yeast cultures can be treated in a variety of ways to produce a supplement for enteric consumption by the host. These treatments include lyophilization or other methods of preserving yeast, in addition to combining the bacteria with carrier agents, such as solutions, solvents, dispersion media, delay agents, emulsions and the like. The use of these agents to prepare supplements is well known in the art. In another embodiment, the transformed yeast are used in the creation of food products, such as fermented milk products like yogurt and kefir, by techniques known to those skilled in the art. As with live lactic acid bacterial cultures in these foodstuffs, the transformed yeast colonize the gut at least transiently and serve to present peptides to the host via the gut lumen.

EXAMPLE 4

Targeting of a peptide to a particular location

The following is provided as an exemplary method to selectively deliver a peptide of this invention to a particular compartment, organ, cell type or location within the body. In this case, nephritis is treated by targeting a peptide to tissues in the kidney of an individual. A sample of a peptide of this invention that displays anti-nephritic activity, CMS030, is obtained from a source in a pure form, such as one of the genetically engineered organisms described in the examples above. This peptide can also be extracted from a biological source such as a tissue sample using techniques familiar to those with skill in the art; it can be synthesized in vitro as well, using any one of the chemical or enzymatic systems for peptide generation available commercially. The peptides in the sample are then linked by covalent bonds via chemical reactions known in the art to low molecular weight (LMW) lysozyme, a commercially available protein moiety that concentrates specifically in renal tissue. Techniques for achieving conjugation of molecules to LMW lysozyme are documented (Folgert *et al.*, Br. J. Pharmcology, 136:1107, 2002). General techniques for conjugating proteins or peptides to one another are also taught in the literature of the field (Fischer *et al.*, Bioconj. Chem., 12:825, 2001). The newly created conjugated peptide sample is then purified away from chemical reagents used in the linking process by chromotography methods such as cation exchange FPLC and/or gradient centrifugation. Once purified, the conjugated peptide is administered to an individual in need of therapy for nephritis. The anti-nephritic CMS030 peptide is preferentially targeted to renal tissue by virtue of the link between it and the LMW lysozyme, which is selectively absorbed and catabolized by cells of the proximal tubules of the kidney. This preferential delivery allows

a greater anti-nephritic effect compared to that of a molar equivalent amount of CMS030 peptide by itself. Inversely, it can reduce the amount of peptide drug required to achieve a certain level of anti-nephritic activity.

EXAMPLE 5

Enhancing the delivery of a peptide to its active site

The following is presented as an exemplary method to increase the delivery of a neuroactive peptide to the brain. A peptide of the present invention that exerts its effects on receptors expressed by neurons of the brain is synthesized by chemical methods known to those with skill in the art. Alternatively, it can be expressed by an engineered microorganism and recovered from a culture of such organisms, as detailed in examples above. Once obtained in a purified form, the peptide is utilized in a series of organic chemical reactions to create a triglyceride ester conjugated moiety, attached to the peptide. The conjugated moiety consists of a quaternary substituted carbon center joined to the peptide of the invention through an amide bond with the terminal carboxyl carbon of the peptide. The other three groups attached to the quarternary carbon center consist of carbon ester linkages to 16 carbon fatty acid chains. The fatty acid chains themselves end in terminal dipeptide group, known as a peptide mask, which makes the chains more hydrophilic and targets them to the blood-brain barrier's endothelial cell membrane specifically. The procedure for this synthesis is explained at length in Patel *et al.*, Bioconjugate Chem., 8(3):434, 1997, and utilizes common reagents and equipment familiar to those with skill in the art.

Once introduced into an individual at a peripheral location, the compound travels throughout the body via the circulatory system, interacting with the endothelial membrane of the blood brain barrier. Step-wise degradation of the dipeptide mask and the lipid chains during the transport of the molecule across the epithelial layer of the blood-brain barrier results in the release of the peptide of the invention into the brain compartment. There the peptide can interact with receptors on the surface of neurons to exert its effect on brain function. The time required for the drug to reach the blood brain barrier and be transported to the brain, with the concomitant degradation of the carrier moiety, alters the kinetics of the drug's activity, creating a more stable and longer lasting effect as compared to the intracerebro ventricular injection of the free peptide.

EXAMPLE 6

Creating peptide formulations that are resistant to enzymatic degradation

The following is provided as an exemplary method for creating a formulation of a biologically active peptide for oral administration that is resistant to the activity of proteases and peptidases found in and along the surface of the digestive tract. A peptide of the present invention that has a desirable biological activity when administered to an individual is synthesized by chemical methods known to those with skill in the art. Alternatively, it can

be expressed by an engineered microorganism and recovered from a culture of such organisms, as detailed in examples above. Once obtained in a purified form, the peptide is utilized in the making of a pharmaceutical formulation for oral administration to a patient. As described in Larionova *et al.* (Int. J. Pharma., 189:171, 1999), the peptide is used in the creation of microparticles with soluble starch and a protease inhibitor, aprotinin, that is a strong inhibitor of a variety of luminally secreted and brush border membrane-bound proteases. Briefly, soluble starch, the protease inhibitor aprotinin and the peptide of the invention are dissolved in an aqueous buffer. The ratios of soluble starch, aprotinin peptide are determined by experimental methods familiar to one with skill in the art; for example, Larionova *et al.* utilized *in vitro* simulated digestion assays to determine the ratios and preparation conditions most effective for the protein used in their study. The aqueous solution is emulsified under mechanical agitation in cyclohexane (1:3 ratio, v/v) containing 5% Span-80, a non-ionic surfactant. A terephthaloyl chloride solution in chloroform is added to the emulsion and stirring is continued 30 minutes, during which the starch molecules are cross-linked with the aprotinin and the peptide. The microparticles created in that process are washed with sequentially with cyclo-hexane, a 95% ethanol solution with 2% v/v Tween 85 detergent, 95% ethanol and water. The microparticles are resuspended in water and lyophilized. The lyophilized compound can be placed into gelatin capsules for oral delivery to the individual in need of treatment.

Once ingested, the compound is released as the gelatin capsule dissolved. The microparticles are broken down in the small intestine by the action of α amylase on the starch molecules, leading to the gradual release of aprotinin and the peptide of the invention. The concurrent release of the potent protease inhibitor aprotinin at the same time and location of the peptide decreases the enzymatic degradation of the peptide and increases the proportion of intact peptide available for absorption through the gut membrane.